THE ROLE OF FLOWER COLOR IN ANGIOSPERM EVOLUTION

EDITED BY: Montserrat Arista, Eduardo Narbona, Justen Whittall, Mani Shrestha and Maria Gabriela Gutierrez Camargo

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THE ROLE OF FLOWER COLOR IN ANGIOSPERM EVOLUTION

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EDITORIAL
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Editorial: The Role of Flower Color in Angiosperm Evolution

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Editorial on the Research Topic

The Role of Flower Color in Angiosperm Evolution

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Although angiosperms exhibit a wide range of variability in floral traits such as shape and size, flower color is a hallmark of angiosperm diversity. Since before Darwin's time, flower color has long been appreciated for its role in pollinator attraction (Sprengel, 1793; Mendel, 1866; Darwin, 1895; Faegri and van der Pijl, 1966; Proctor and Yeo, 1973). However, over the past few decades, a growing body of evidence suggests that flower color can be molded by a diversity of selective pressures. The rapid accumulation of flower color studies has spurred several thorough reviews (Winkel-Shirley, 2001; Koes et al., 2005; Rausher, 2008; Sobel and Streisfeld, 2013; Narbona et al., 2018; Sapir et al., 2021), but here we present the largest collection of investigations specifically focused on the role of flower color in angiosperm evolution.

This Research Topic is composed of 28 studies on the role of flower color in angiosperm evolution. These contributions include species living on nearly all continents plucked from most major branches of the angiosperm tree of life (**Figure 1**). Investigations span traditional scales in biology, from gene expression and biochemical profiles to pollinator perception and community assembly. Evolutionarily, studies range from within species flower color polymorphisms to macroevolutionary patterns of flower color evolution within and among genera. Ecologically, investigations span a diversity of plant communities including neotropical savannas, temperate serpentine seeps, subtropical mountains, and tropical dry forests.

Fundamentally, flower color depends on the underlying pigments and recent studies have begun to decipher the genetic basis of this pigment production. The most prevalent and variable pigments in flowers are the anthocyanins, which originate from the flavonoid biosynthetic pathway (Tanaka et al., 2008). Branches of this pathway produce other flavonoid compounds (e.g., flavonols, flavones, isoflavones, proanthocyanins, and catechins) that protect plants against a variety of environmental stressors such as pathogens, herbivores, drought, extreme temperatures, ultraviolet (UV) radiation, etc. (Pollastri and Tattini, 2011; Falcone Ferreyra et al., 2012; Jiang et al., 2016). Thus, certain floral colors not only affect pollinator attraction (or avoidance), but may also determine resistance to biotic and abiotic stresses (Strauss and Whittall, 2006; Landi et al., 2015).



FIGURE 1 | Examples of species included in our Research Topic "*The Role of Flower Color in Angiosperm Evolution*." **(A)** *Senna rugosa* (Fabaceae) using UV (left) and conventional photography (right) (Brazil, Mariah di Stasi). **(B)** Flower color variation in *Campanula americana* (Campanulaceae, USA, Matthew Koski). **(C)** *Aristolochia fimbriata* (Aristolochiaceae, Colombia, Natalia Pabón-Mora). **(D)** *Jaborosa rotacea* (Gesneriaceae, Argentina, Marcela Moré). **(E,F)** White and pink flowers of *Silene littorea* plants (Caryophyllaceae, Spain, Eduardo Narbona). **(G,H)** *Potentilla plattensis* and *Argentina anserine* (Rosaceae, USA, Matthew Koski). **(I)** *Gentiana flavomaculata* (Gentianaceae, Taiwan, Chun-Neng Wang). **(J)** *Caladenia fulva* (Orchidadeae, Australia, Ann Lawrie). **(K,L)** Flower color morphs of *Erythranthe discolor* (Phrymaceae, USA, Naomi Fraga and Dena Grossenbacher). **(M,N)** Flower color morphs of *Mandevilla sanderi* (Apocynaceae, The Netherlands, Doekele Stavenga). **(O)** *Meconopsis horridula* (Papaveraceae, China, Anke Jenstsch). **(P)** *Caladenia caerula* (Orchidaceae, Australia, Mani Shrestha). **(Q)** *Lobelia rhombifolia* (Campanulaceae, Australia, Mani Shrestha). **(R)** Flower color variation in *Tibetia yunnanensis* (Leguminosae, China, Klaus Lunau). **(S)** Plants of *Eriophyllum lanatum* (Asteraceae) and *Clarkia concinna* (Onagraceae) coexisting (USA, Gerardo Arceo-Gómez). **(T)** Flower color morphs of *Lysimachia arvensis* coexisting (Primulaceae, Spain, Montserrat Arista).

THE GENETICS AND BIOCHEMISTRY OF ANTHOCYANIN PRODUCTION

The anthocyanin biosynthetic pathway is mostly regulated at the transcriptional level (Albert et al., 2014). In this volume, Muñoz-Gómez et al. studied the regulatory gene evolution in the anthocyanin biosynthetic pathway among the Aristolochiaceae, a family with a high floral diversity exhibiting elaborate color patterns. The authors conclude that the anthocyanin biosynthetic pathway and its regulatory genes are largely conserved across the family, and color variation is primarily determined by differences in gene expression. Stavenga et al. studied the pH dependence of flavonoid absorption spectra of two Papaveraceae species, *Papaver dubium* (red) and *Meconopsis cambrica* (orange), and white and red varieties of *Mandevilla sanderi*. The authors found that the absorption spectrum (i.e. colour) of anthocyanins can be dependent on the pH of the vacuole, however, not all flavonoids respond similarly to changes in vacuolar pH. Color diversity is also remarkably high in Gesnerioideae and Ogutchen et al. have elegantly shown that this is due to the expanded use of the anthocyanin biosynthetic pathway. In this family, the anthocyanin biosynthetic pathway includes the deoxyanthocyanin branch, which is rarely functional in angiosperms. Ogutchen et al. also call for a better understanding of the link between the biochemical basis of flower color and the visual perception of the primary pollinators. At the microevolutionary scale, Sánchez-Cabrera et al. identified several anthocyanin biosynthetic pathway loci likely involved in the shift in flower color in the orange/blue polymorphic *Lysimachia arvensis*. Further, these authors found differential expression of two genes (*F3′5′H* and *DFR*) in the anthocyanin biosynthetic pathway. The biochemical analysis of color morphs was consistent with the transcriptome data indicating that the shift from blue to orange petals is caused by a change from primarily malvidin to largely pelargonidin forms of anthocyanins (Sánchez-Cabrera et al.). Authors discuss that both the decreased expression of *F3'5'H* in orange petals and the differential expression of two distinct copies of *DFR*, which also exhibit amino acid changes in the color-determining substrate specificity region, strongly correlate with the blue to orange transition. Collectively, these studies have revealed the complexity and nuance of the pigment biochemistry, yet the consistent role of gene expression changes in the anthocyanin biosynthetic pathway underlying the diversity of flower colors.

HOW IS COLOR PERCEIVED?

Flower color is in the eye of the beholder. Color perception depends on the sensory abilities of different groups of pollinators (van der Kooi et al., 2021). Flower color variation can arise when pollinators with diverse sensory systems drive fitness differences between flower color variants (Koski, 2020). Thus, in order to understand the selective role of pollinators in flower color, it is necessary to incorporate their color perception. Garcia et al. shows that experienced pollinators often make correct decisions about the presence of rewards based on flower color, but this is not the case for inexperienced pollinators. These authors concluded that color cannot be considered an inherent trait because its interpretation by an animal's brain is frequently context-dependent.

Although most studies of color perception and foraging decisions have been traditionally based on *Apis mellifera*'s sensory system (e.g., Giurfa et al., 1994; Dyer et al., 2008; Rohde et al., 2013), we are in dire need of experimental studies on the sensory capabilities of non-*Apis* bee pollinators. In this volume, Koethe et al. have carried out a comparative study of food source selection between two stingless bee species and honeybees. These three species reacted similarly to color, but the variation among them could be the result of adaptations to the bees' respective habitat and morphological constraints. Thus, habitat traits can influence color perception by pollinators. In a similar conceptual framework, Martins et al. studied how seasonal changes in the leaf-background colouration of Brazilian savanna communities affect the perception of flower color contrasts by bees. They found that background coloration affected flower contrasts, favoring flower conspicuousness to bees according to the season and providing new insights regarding the temporal patterns of plant–pollinator interactions.

Dyer et al. reviewed the old concept of the rarity of blue flowers. They found that short wavelength reflecting blue flowers are indeed frequent in nature when considering the color vision of bees and they point out that competition for pollinators may drive the evolution of blue flowers. Coimbra et al. used the visual system of bees to test the generalization of the bee-avoidance hypothesis proposed to explain why bird pollinated flowers tend to be red. Their results suggest that bee sensory exclusion *via* color signals is exclusive to bird flowers, while non-bee, insect flowers might use other sensory channels to exclude bees. In another study, Whitney et al. analyzed how flower color variation within plant populations of bee- and hummingbird-pollinated plant species is perceived by their specific pollinators. They found that bees sensed equal color variation within species from the two pollination systems, but birds perceived more color variation in bird-pollinated flowers than in bee-pollinated flowers.

FLOWER COLOR VARIATION: A BROAD SCALE APPROACH

Phylogenetically controlled studies at the community level have repeatedly found that flower color tends to show weak phylogenetic signal, reflecting an underlying pattern of evolutionary divergence (e.g., McEwen and Vamosi, 2010; Muchhala et al., 2014; Ortiz et al., 2021). For example, Tai et al. studied flower color signaling in Taiwan and found that although high altitude floras tend to be phylogenetically clustered, their flower colors exhibited only weak phylogenetic signal. Thus, they suggest that flower color signaling was mainly influenced by color preferences of key bee pollinators. Most studies on the phylogenetic signal of flower color focus on the predominant color exhibited by the flowers, although in many species flowers are not uniform showing contrasting colors (i.e., color patterns) that are perceived by pollinators (Hempel de Ibarra et al.,

2015). Some color patterns include reflection of UV light that is perceived by pollinators that have UV photoreceptors (van der Kooi et al., 2021). When floral color patterns in the ultraviolet spectrum (UV patterns) are investigated, contrasting results have been found. Tunes et al. studied patterns of floral UV reflectance in plants from a Neotropical savanna. They tested the roles of phylogenetic relatedness and pollinator mediated selection on the distribution of UV floral patterns. They confirmed that phylogenetic relatedness constrains the diversity of floral UV patterns, however, the distribution of floral UV-features could not be ascribed to a single ecological or evolutionary factor. This study calls for a deeper understanding of ecological and evolutionary processes involved when interpreting floral UV color diversification. Some flowers of the Potentilleae tribe (Rosaceae) have petals with UV patterns, whereas others show human-visible patterns or uniform petal color. Koski investigated the evolutionary transition between patterned and non-patterned petals and found that the presence of UV and human-visible patterns evolved independently from one another. He also found that the evolution of human-visible patterns was associated with the evolution of larger flowers, supporting the hypothesis that nectar or pollen guides are more likely to evolve in larger-flowered species. In another study, Roguz et al. explored the evolution of flower color in *Iris*, a genus displaying a huge diversity of flower color and color patterns among and within species. They found that the most recent common ancestor likely had entomophilous, monochromatic flowers.

The fate of new color variants depends on a diversity of evolutionary forces. Flower color can affect pollinator attraction and, when investigated in a community context, flower color can mediate different types of interactions among co-flowering species, such as competition, facilitation or mimicry (Ghazoul, 2006; Johnson and Schiestl, 2016; Kemp et al., 2019). The abundance and species richness of the local habitat may also influence the type or strength of ecological interactions among species. Co-flowering species with similar flower colors may compete for pollinator services; selection could thus favor differentiation to improve pollinator recognition and fidelity (Gumbert et al., 1999; McEwen and Vamosi, 2010). Competition for pollinators would result in a pattern of phylogenetic over-dispersion of floral color among co-flowering species (Sargent and Ackerly, 2008). Alternatively, similarity in flower color may result from selection for standardization of the signals that improves recognition by pollinators and increases their visitation rates. LeCroy et al. studied 14 serpentine seep communities in California differing in species richness and size. In smaller-sized communities they found that competitive exclusion could be a dominant process shaping lower species richness, but this process is less detectable in larger, more speciose communities. Similarly, Moré et al. explored the potential importance of pollinators as drivers of floral color diversification in the genus *Jaborosa* taking into account the perceptual abilities of their pollinators (i.e., nocturnal hawkmoths versus diurnal saprophilous flies) with a geographical perspective. This study found that the ability of plants that colonized the newly formed environments during Andean orogeny and the ecological changes that followed were concomitant with transitions in flower color as perceived by different pollinator functional groups. Further, this study suggests that habitat and pollination are linked to the history of a plant's lineage.

FLOWER COLOR VARIATION AT THE MICROEVOLUTIONARY SCALE

From a microevolutionary point of view, intraspecific variation in floral color is widely distributed (Narbona et al., 2018). Flower color may vary continuously or discretely, the latter situation being much rarer. Evolutionary mechanisms explaining the maintenance of intraspecific flower color variation include both biotic and abiotic factors, although other processes can also play an important role (Narbona et al., 2018). Trunschke et al. reviewed studies addressing selection on continuous flower color variation in the context of pollinator interactions. They suggest that evidence for significant pollinator-mediated selection is surprisingly limited among existing studies. In fact, most of the current understanding of flower color evolution arises from variation between discrete color morphs, where selection by pollinators is usually one of the most important factors involved in color variation. Selective foraging by pollinators for specific color morphs is frequently reported (Ortiz et al., 2015). These studies focus on the most common pollinators such as bees or birds, while selection by other groups is rarely reported.

Two studies in this volume have investigated microevolutionary flower color variation in South-African species pollinated by less-commonly-reported pollinator groups. Ellis et al. studied patterns of color distribution of discrete white and orange daisy species pollinated by bombyliids. They found that the dominant pollinator in orange communities has strong preferences for orange flowers while the dominant pollinator in white communities exhibited an innate preference for white flowers. These findings demonstrate that landscape-level flower color turnover is likely shaped by a strong qualitative geographic mosaic of bee-fly pollinators with divergent color preferences. Similarly, Johnson et al. found that patterns of color variation in *Drosera cistiflora*, a species pollinated by beetles, are associated with different pollinator communities. Given that these beetle species discriminate among color forms (von Witt et al., 2020), the authors conclude that beetle pollinators are a significant factor in the evolution of *D. cistiflora* flower color. This is one of the first reports of flower color selection by beetles (see Streinzer et al., 2019).

Although direct selection on flower color by pollinators has been widely explored, indirect selection on correlated traits is rarely reported (e.g., Gómez, 2000). Other floral traits can be correlated with flower color and thus, to understand the role of pollinators as selective agents on flower color it is necessary to consider such associations. The number of flowers per plant, a component of floral display, is one of the most important traits affecting pollinator attraction, and its association with flower color has been studied in *Silene littorea* (Rodríguez-Castañeda et al.) and *Medicago sativa* (Brunet et al.). Both studies show significant correlations

between flower color and floral display and demonstrate significant directional selection on floral display that indirectly selects for flower color. These examples of correlational selection can partly explain flower color evolution in *M. sativa* and the maintenance of the flower color polymorphism in *S. littorea*.

The synthesis of floral pigments can also have pleiotropic effects on defensive plant compounds and consequently herbivores can play a role in flower color variation (Strauss and Whittall, 2006). Specifically, anthocyanin pigments in floral tissues can influence herbivore preference and performance (Irwin et al., 2003; Frey, 2004). In this volume, Sobral et al. found evidence of transgenerational effects of herbivory on flower color variation in *Raphanus sativus*. Epigenetic modifications due to herbivory influences the proportion of plants with anthocyanins in the following generation showing a link between biotic ecological interactions across generations and plasticity in flower color; with some exceptions this phenomenon is virtually undescribed in natural plant populations.

Abiotic factors also play an important role in flower color variation (Strauss and Whittall, 2006). Water availability, temperature and solar radiation can select flowers with higher pigment concentrations either directly or indirectly, giving rise to geographical patterns of flower color variation (Dalrymple et al., 2020). The role of abiotic factors on flower color variation is more frequently reported and, in this volume, it has been addressed in two monkeyflowers– *Erythranthe discolor* and *Diplacus mephyticus* (Grossenbacher et al.), *Campanula americana* (Koski and Galloway), and *Drosera cistifolia* (Johnson et al.). Drought stress is one of the most important factors predicting flower color across geographic ranges (Warren and Mackenzie, 2001; Arista et al., 2013). Grossenbacher et al. found a higher frequency of anthocyanin producing morphs in populations with reduced precipitation for two monkeyflower species as a consequence of the protective role of anthocyanins. However, after accounting for phylogeny, there was no evidence that drought stress leads to a macroevolutionary pattern in flower color across monkeyflowers. In *Campanula americana* local temperature appears to shape the geographical pattern of flower color intensity, although the genetic population structure seems to be driven by historical effects, an important factor rarely considered (Koski and Galloway). Conversely, other rarely studied, potential abiotic selective agents (such as soil type) do not seem to be related to the geographic pattern of color variation in *Drosera cistifolia* (Johnson et al.). The role of abiotic factors on flower color variation has also been reported in this volume. Peach et al. studied the effect of UV radiation on flower color variation in *Clarkia unguiculata* and found that populations growing in areas of high UV radiation showed higher anthocyanin concentration, a pattern previously reported (Del Valle et al., 2015). However, contrary to expectations, UV-absorbing floral patterns did not have a direct "pollen protection" function, highlighting the need for research on a wider range of taxa to understand the role of anthocyanins in flower protection against UV radiation [see Kay et al. (2020)]. These studies addressing the role of abiotic factors affecting flower color variation highlight the protective role of pigments under some conditions of abiotic stress, but also reveal the multifaceted evolutionary and ecological complexity underlying natural flower color variation.

Other less known factors involved in the maintenance of intraspecific flower color variation are also reported in this volume. Jiménez-López et al. explored the role of selfing as a way to maintain the flower color polymorphism in *Lysimachia arvensis*. They found that in Mediterranean populations, both biotic and abiotic factors select against one of the color morphs, but that increased selfing in this morph preserves the color variation within populations. One consequence of this mating system by flower color interaction is a decrease in genetic variation that could have macroevolutionary consequences.

Flower color also shows intrafloral variation giving rise to complex patterns that can attract and guide bee pollinators. Aguiar et al. studied intrafloral color variation in *Cattleya walkeriana*, and found a centripetally increasing spectral purity within the flowers of this bee pollinated orchid species. This intrafloral variation was unrelated to the development of floral structures, suggesting an important role of pollinator selection in the modularization of flower color.

Lastly, the importance of taking flower color into account in conservation strategies is highlighted in this special topic. Orchidaceae is one of the most threatened plant families, as it has very large numbers and proportions of endangered species worldwide. Intraspecific flower color variability in the endangered orchid *Caladonia fulva* has been suggested to be the result of hybridization with *C. reticulata*. Genetic and breeding studies by Basist et al. clearly shows that *C. fulva* is a flower color polymorphic species and conservation of the color variants is essential to maintain genetic diversity.

In this Research Topic, we present a modern synthesis of flower color studies. Clearly, understanding the evolution of flower colors in angiosperms requires a diversity of perspectives across scales, both ecological and evolutionary, from the molecular, biochemical, physiological, anatomical, organismal, population, and community levels. These studies show that flower color in plant populations and plant communities are often the result of a combination of biotic and abiotic selective pressures and are highly context dependent. They clearly indicate the need to incorporate new approaches and innovative methodologies when studying flower color evolution.

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All authors contributed to the article and approved the submitted version.

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REFERENCES

Albert, N. W., Davies, K. M., Lewis, D. H., Zhang, H., Montefiori, M., Brendolise, C., et al. (2014). A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *Plant Cell* 26, 962–980. doi: 10.1105/tpc.113.122069

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

Darwin, C. (1895). *The Effects of Cross and Self-Fertilisation in the Vegetable Kingdom*. New York, NY: D. Appleton.

Del Valle, J. C., Buide, M. L., Casimiro-Soriguer, I., Whittall, J. B., and Narbona, E. (2015). On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (*Silene littorea*). *Front. Plant Sci.* 6:939. doi: 10.3389/fpls.2015.00939

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp Physiol. A* 194:617. doi: 10.1007/s00359-008-0335-1

Faegri, K., and van der Pijl, L. (1966). The principles of pollination ecology. *Toronto Oxford* 7, 157–159. doi: 10.1016/B978-0-08-023160-0.50020-7

Falcone Ferreyra, M. L., Rius, S. P., and Casati, P. (2012). Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front. Plant Sci.* 3:222. doi: 10.3389/fpls.2012.00222

Frey, F. M. (2004). Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacacea). *Evolution* 58, 2426–2437. doi: 10.1111/j.0014-3820.2004.tb00872.x

Ghazoul, J. (2006). Floral diversity and the facilitation of pollination. *J. Ecol.* 94, 295–304. doi: 10.1111/j.1365-2745.2006.01098.x

Giurfa, M., Núñez, J., and Backhaus, W. (1994). Odour and colour information in the foraging choice behaviour of the honeybee. *J. Comp. Physiol. A* 175, 773–779. doi: 10.1007/BF00191849

Gómez, J. M. (2000). Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *J. Evol. Biol.* 13, 689–699. doi: 10.1046/j.1420-9101.2000.00196.x

Gumbert, A., Kunze, J., and Chittka, L. (1999). Floral colour diversity in plant communities, bee colour space and a null model. *Proc. Royal Soc. London B* 266, 1711–1716. doi: 10.1098/rspb.1999.0836

Hempel de Ibarra, N., Langridge, K. V., and Vorobyev, M. (2015). More than colour attraction: behavioural functions of flower patterns. *Curr. Opin. Insect. Sci.* 12, 64–70. doi: 10.1016/j.cois.2015.09.005

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-9658(2003)0841733:TROHIT2.0.CO;2

Jiang, N., Doseff, A. I., and Grotewold, E. (2016). Flavones: from biosynthesis to health benefits. *Plants* 5:27. doi: 10.3390/plants5020027

Johnson, S. D., and Schiestl, F. P. (2016). *Floral Mimicry*. Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780198732693.001.0001

Kay, K. M., Jogesh, T., Tataru, D., and Akiba, S. (2020). Darwin's vexing contrivance: a new hypothesis for why some flowers have two kinds of anther. *Proc. R. Soc. B* 287:20202593. doi: 10.1098/rspb.2020.2593

Kemp, J. E., Bergh, N. G., Soares, M., and Ellis, A. G. (2019). Dominant pollinators drive non-random community assembly and shared flower colour patterns in daisy communities. *Ann. Bot.* 123, 277–288. doi: 10.1093/aob/mcy126

Koes, R., Verweij, W., and Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* 10, 236–242. doi: 10.1016/j.tplants.2005.03.002

Koski, M. H. (2020). The role of sensory drive in floral evolution. *New Phytol.* 227, 1012–1024. doi: 10.1111/nph.16510

Landi, M., Tattini, M., and Gould, K. S. (2015). Multiple functional roles of anthocyanins in plant-environment interactions. *Environ. Exp. Bot.* 119, 4–17. doi: 10.1016/j.envexpbot.2015.05.012

McEwen, J. R., and Vamosi, J. C. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proc. Royal Soc. B* 277, 2957–2965. doi: 10.1098/rspb.2010.0501

Mendel, G. (1866). Versuche über Pflanzen-hybriden. *Verh. Natforsch. Ver. Brünn* 4, 3–47. doi: 10.5962/bhl.title.61004

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Ortiz, P. L., Berjano, R., Talavera, M., Rodríguez-Zayas, L., and Arista, M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: how is the red morph maintained in Mediterranean environments? *Persp. Plant Ecol. Evol. Syst.* 17, 142–150. doi: 10.1016/j.ppees.2015.01.004

Ortiz, P. L., Fernández-Díaz, P., Pareja, D., Escudero, M., and Arista, M. (2021). Do visual traits honestly signal floral rewards at community level? *Funct. Ecol.* 35, 369–383. doi: 10.1111/1365-2435.13709

Pollastri, S., and Tattini, M. (2011). Flavonols: old compounds for old roles. *Ann. Bot.* 108, 1225–1233. doi: 10.1093/aob/mcr234

Proctor, M., and Yeo, Y. (1973). *The Pollination of Flowers*. Glasgow: William Collins.

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Intern. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Sapir, Y., Gallagher, M. K., and Senden, E. (2021). What maintains flower colour variation within populations?. *Trends Ecol. Evol.* 36, 507–519. doi: 10.1016/j.tree.2021.01.011

Sargent, R. D., and Ackerly, D. D. (2008). Plant–pollinator interactions and the assembly of plant communities. *Trends Ecol. Evol.* 23, 123–130. doi: 10.1016/j.tree.2007.11.003

Sobel, J., and Streisfeld, M. A. (2013). Flower color as a model system for studies of plant evo-devo. *Front. Plant Sci.* 4:321. doi: 10.3389/fpls.2013.00321

Sprengel, C. K. (1793). "Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen. English translation by P. 1996," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 3–43. doi: 10.5962/bhl.title.13716

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder, and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (*Anemone pavonina*, Ranunculaceae) in Northern Greece. *J. Comp. Physiol. A* 205, 735–743. doi: 10.1007/s00359-019-01360-2

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Ann. Rev. Entomol.* 66, 435–461. doi: 10.1146/annurev-ento-061720-071644

von Witt, C. G., Anderson, B., Durbach, I. N., and Johnson, S. D. (2020). Breeding systems of floral colour forms in the *Drosera cistiflora* species complex. *Plant Biol.* 22, 992–1001. doi: 10.1111/plb.13159

Warren, J., and Mackenzie, S. (2001). Why are all color combinations not equally represented as flower-color polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Winkel-Shirley, B. (2001). Flavonoid biosynthesis: a colourful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126, 485–493. doi: 10.1104/pp.126.2.485

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A Comparative Study of Food Source Selection in Stingless Bees and Honeybees: Scent Marks, Location, or Color

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In social bees, the choice of food sources is based on several factors, including scent marks, color, and location of flowers. Here, we used similar setups, in which two stingless bee species, *Melipona subnitida* and *Plebeia flavocincta*, and the Western honeybee, *Apis mellifera*, were tested regarding the importance of chemical cues, color cues, and location-dependent cues for foraging behavior. It was determined whether workers chose food sources according to (1) scent marks deposited by conspecifics, (2) the color hue of a food source, (3) the trained location or the proximity of a food source to the hive. All three species preferred the scent-marked over an unmarked feeder that was presented simultaneously, but *M. subnitida* showed a weaker preference compared to the other species. When trained to blue feeders all three bee species preferred blue, but *A. mellifera* showed the strongest fidelity. The training to yellow feeders led to less distinct color choices. Only workers of *M. subnitida* mostly orientated at the training position and the close proximity to the nest. Whether the distance of a feeding site influenced the choice was dependent on the tested parameter (color or scent marks) and the species. Workers of *M. subnitida* preferably visited the feeder closer to the nest during the scent mark trials, but choose randomly when tested for color learning. Worker honeybees preferred the closer feeding site if trained to yellow, but not if trained to blue, and preferred the more distant feeder during the scent mark trials. Workers of *P. flavocincta* preferred the closer feeder if trained to blue or yellow, and preferred the more distant feeder during the scent mark trials. The disparity among the species corresponds to differences in body size. Smaller bees are known for reduced visual capabilities and might rely less on visual parameters of the target such as color hue, saturation, or brightness but use scent cues instead. Moreover, the dim-light conditions in forest habitats might reduce the reliability of visual orientation as compared to olfactory orientation. Honeybees showed the most pronounced orientation at floral color cues.

Keywords: eusocial bees, chemical cues, color cues, location-dependent cues, foraging behavior

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INTRODUCTION

Foraging bees use visual and olfactory cues to find and select food sources and deploy innate or learned preferences to detect flowers (Lunau and Maier, 1995; Dyer et al., 2016). Primarily, a forager's choice is biased by innate preferences for particular colors, shapes, and odors (Menzel, 1967; Giurfa et al., 1995; Lehrer et al., 1995; Lunau et al., 1996; Gumbert, 2000; Biesmeijer and Slaa, 2004; Raine and Chittka, 2007; Howard et al., 2019). These innate preferences differ among species. In several experiments, preferences for specific hues and saturation of colors could be found for honeybees and bumble bees (Lunau, 1990; Giurfa et al., 1995; Lunau et al., 1996; Papiorek et al., 2013; Rohde et al., 2013), while stingless bees sparsely show preferences for color hue or saturation (Spaethe et al., 2014; Dyer et al., 2016; Koethe et al., 2016, 2018).

With increasing foraging experience, initial individual preferences may be either consolidated or modified through associative learning (Gumbert, 2000; Sánchez et al., 2008; Roselino et al., 2016). For instance, species-specific chemical footprints deposited by bees while landing on and manipulating flowers indicate the recent presence of a forager to subsequent visitors (Hrncir et al., 2004; Jarau et al., 2004; Eltz, 2006; Saleh and Chittka, 2006; Witjes et al., 2011). An initial attraction toward the familiar scent of conspecifics (Schmidt et al., 2005) may be reinforced when individuals learn to associate the footprints with high reward levels or reversed when scent marks indicate depleted flowers (Saleh and Chittka, 2006; Roselino et al., 2016).

Learning and memory play a major role in bee foraging, enabling the repeated visit to sustainable food sources (Breed et al., 2002; Reinhard et al., 2004, 2006; Jesus et al., 2014), flower constancy (Free, 1963; Biesmeijer and Toth, 1998; Slaa et al., 1998, 2003), and the discovery of new patches of known food plants (Biesmeijer and Slaa, 2004). In addition to memorizing scent and location of resources (Reinhard et al., 2004, 2006), bees learn both color and position of landmarks, which facilitates the orientation toward food sources and the nest (Cartwright and Collett, 1983; Cheng et al., 1986, 1987; Chittka et al., 1995; Menzel et al., 2005). However, species differ concerning their learning ability (Pessotti and Lé'Sénéchal, 1981; Mc Cabe et al., 2007), which might be associated with differences in life-history and ecological traits among bee species, such as longevity of individuals (Ackerman and Montalvo, 1985), the degree of floral specialization (Cane and Snipes, 2006), and food niche-breath (Biesmeijer and Slaa, 2006).

In eusocial bees, including the stingless bees (Meliponini), bumble bees (Bombini), and honeybees (Apini), food source selection is not only based on individual foraging preferences, but relies to a large extent on social information. On their return to the nest, foragers transmit olfactory and gustatory information about the exploited food source to nestmates, which biases the subsequent food choice of the receivers (Farina et al., 2005, 2007; Mc Cabe and Farina, 2009). Moreover, returning foragers of many species announce the existence of lucrative food sources through thoracic vibrations (stingless bees: Lindauer and Kerr, 1958; Esch et al., 1965; Barth et al., 2008; Hrncir and Barth, 2014; honeybees: Esch, 1961; Waddington and Kirchner, 1992; Hrncir et al., 2011). Inactive individuals may use these mechanical signals for their decision of whether to engage in foraging or to remain in the nest. In addition, foragers of some eusocial bee species guide the recruits to the location of the exploited food patch. Honeybees (all species) use an elaborated dance language (waggle dance) communicating information about distance, direction, and quality of foraging sites (Von Frisch, 1967; Dyer, 2002). Stingless bees (few species), in contrast, lay polarized trails of species-specific pheromone marks that guide recruits with high precision toward the goal (Lindauer and Kerr, 1958; Schmidt et al., 2003; Nieh et al., 2004; Barth et al., 2008; Jarau, 2009). At the food patch, foraging choices are influenced by field-based social information, like olfactory footprints and the visual presence of con- or heterospecific foragers (Slaa et al., 2003). Depending on the composition of the foraging community at the food patch, these passively provided cues may cause local enhancement or local inhibition (Slaa and Hughes, 2009). Thus, food source selection in eusocial species is based on a complex interplay between individual preferences and social information.

Differences among social bee species regarding ecological (habitat, food niche), physiological (learning ability, visual capacity, color vision), and behavioral features (innate preferences, foraging strategy, recruitment mechanism) may result in differences concerning the parameters used in foraging decisions. With more than 500 described species, stingless bees (Meliponini) are the most speciose group of eusocial bees with very diverse characteristics regarding body size, colony size, nesting biology, brood cell arrangement, queen production, foraging strategies, and recruitment mechanisms (Michener, 1974, 2013; Johnson, 1983; Wille, 1983; Engels and Imperatriz-Fonseca, 1990; Roubik, 2006; Barth et al., 2008). Given this biological diversity, we can expect differences concerning the mechanisms of food source selection among species. In the present study, we investigated the food source selection by two stingless bee species, *Melipona subnitida* and *Plebeia flavocincta*, and the Western honeybee, *Apis mellifera*. Since stingless bees show only weak preferences for colors compared to other bee species (Dyer et al., 2016; Koethe et al., 2016, 2018), alternative parameters could be of importance for foraging choices. Of interest were the roles of scent marks (olfactory footprints), the color, and the location of a food source. *Melipona* species are known to mark food sources with olfactory footprints (Jarau, 2009; Roselino et al., 2016). For *P. flavocincta*, no specific information concerning scent communication is available so far (Aguilar et al., 2005). However, given that all bee species studied to this moment deposit chemical footprints at food sources (Goulson et al., 1998; Eltz, 2006; Yokoi et al., 2007; Jarau, 2009; Witjes et al., 2011), scent cues can also be postulated for this meliponine species. *A. mellifera* is known for marking food sources directly (Giurfa and Nunez, 1992).

The aim was to analyze how the three investigated social bee species use the parameters color, scent marks, or location differently during the colony foraging processes. We test the hypothesis that these bees possess a hierarchy in the use of the parameters color, scent marks, and location of flowers. We expect honeybees to rely more on color cues than the two stingless bee species. For the two stingless bee species, we assume that they follow scent markings of conspecifics more reliable than

honeybees. Since small stingless bee might exploit nectarrich flowers by repeated visits to the same individual flower, we assume that the location of the flower is of higher importance in the smaller bees.

MATERIALS AND METHODS

This study is part of a research project on color preferences in stingless bees conducted in Australia and Brazil (Köthe, 2019).

Study Site and Bee Species

The foraging behavior of the stingless bee species was investigated at the Brazilian Federal University at Mossoró (Universidade Federal Rural do Semi-Árido), located in the Brazilian tropical dry forest, the Caatinga at 5°12′13.3″S 37°19′44.8″W. For our experiments, we used two stingless bee species native to the study region, *M. subnitida* (six colonies) and *P. flavocincta* (one colony) (Zanella, 2000; Imperatriz-Fonseca et al., 2017). *P. flavocincta* is the smallest bee with less than 5 mm body length (Cockerell, 1912), *M. subnitida* is intermediate with 7.5–8.5 mm (Schwarz, 1932), and *A. mellifera* is the largest with more than 11 mm (Amiet and Krebs, 2012). Colonies of the stingless bee species were kept in wooden nest-boxes at the university's meliponary (Meliponário Imperatriz) and were freely foraging. The foraging behavior of the Western honeybee, *A. mellifera*, was studied at the botanical garden of the Heinrich Heine University Düsseldorf, Germany at 51°11′10.7″N 6°48′14.1″E. Foragers of five nests were trained to participate in the experiment. The colony size of the three tested species differs and ranges from several thousand individuals (20.000–80.000) in a single colony of *A. mellifera* to several hundred (up to 1000) in *M. subnitida* (Wilson, 1971; Michener, 1974). For *P. flavocincta*, colony size has not been determined yet, but in other *Plebeia* species, colony size has been shown to range from 2.000 to 3.000 individuals (Roldão-Sbordoni et al., 2018).

The reasons for conducting the study on stingless bees and honeybees at different study sites were as follows: Most experimental research in Western honeybees has been done in Europe and Australia, excluding the Africanized honeybees available in Mossoro. Moreover, *A. mellifera* is not native in South America. Thus, direct comparison with literature data is easier when working with European Western honeybees, although direct comparison of foraging strategies in stingless bees and honeybees in the same habitat might also yield interesting results (Roubik and Buchmann, 1984). The origin of the Western honeybee is in the Middle East or Africa (Han et al., 2012) and Western honeybees have developed adaptations to get along with temperate climates (Han et al., 2012).

Bee Training

For all tests and bee species, the training was identical. Workers of all three species were trained to mass feeders offering sugar solution (50%) affixed to tripods. The training to the mass feeders started at the respective nest's entrance. After more than 10 workers regularly foraged at the feeder, it was moved in short steps (∼1 m) away from the nest until a distance of 15 m (site 1) or 17 m (site 2) was reached. Once at the final feeding site, the mass feeder was replaced by a colored gravity feeder (10 cm diameter, 5 cm height) that was used during the experiment. The gravity feeders were either blue (edding permanent spray RAL5010 enzianblau, edding International GmbH, Ahrensburg, Germany) or yellow (only for the color test; edding permanent spray RAL 1037 sonnengelb, edding International GmbH, Ahrensburg, Germany). The colors were measured using spectrometer analysis (USB4000 miniature fiber optic spectrometer, Ocean Optics GmbH, Ostfildern, Germany) at an angle of 45° using a UV-NIR deuterium halogen lamp (DH-2000-BAL, Ocean Optics GmbH), which was connected to the spectrometer by a UV–VIS fiber optic cable (Ø 600 μm, QR600-7-UV 125 BX, Ocean Optics GmbH). To calibrate the spectrometer, a black standard (black PTFE powder, Spectralon diffuse reflectance standard SRS-02-010, reflectance factor of 2.00%, Labsphere, Inc., North Sutton, NH, United States) and a white standard (white PTFE powder, Spectralon diffuse reflectance standard SRS99-010, reflectance factor of 99.00%, Labsphere, Inc., North Sutton, NH, United States) were used (**Supplementary Figure S1**). After the workers accepted the colored gravity feeder (henceforth "feeder"), a training period of 30 min started, in which the bees were allowed to forage *ad libitum* (approximate number of foragers during training phase*: M. subnitida* ≈ 10 individuals; *P. flavocincta* ≈ 30–50 individuals, *A. mellifera* ≈ 30–50 individuals). Workers were not marked during the training to keep the disturbance at the feeder to a minimum. Hence, no discrimination between experienced and inexperienced workers was possible.

Experiments

Testing the Impact of Scent Marks

We conducted experiments investigating the influence of scent marks deposited at the training feeder on the choice behavior of foragers. For this experimental series, we used only blue-colored feeders. In total, we performed three trials with each bee species. In preliminary studies, this approach turned out to be most reasonable for comparative studies between these bee species. Each trial consisted of three sets of a 30-min training phase and a subsequent 5-min test phase, switching the feeder positions in pseudo-randomized order (SM1–SM3; **Supplementary Table S1**). After the training phase, we offered the incoming bees both the training feeder (scent-marked) and a clean blue-colored feeder (unmarked), one at each feeding site (**Supplementary Table S1**). During this test phase, both feeders contained sugar solution (50%). In total, we performed three trials of this experimental series with each bee species. A trial consisted of three pairs of a 30-min training phase and a 5-min test phase intermitted by 30-min training phases (SM1–SM32; **Supplementary Table S1**), switching the feeder positions in pseudo-randomized order. The three different bee species (*A. mellifera*, *M. subnitida*, and *P. flavocincta*) were tested separately. Workers that visited the feeder were either marked with nail polish on their first visit (*A. mellifera* and *M. subnitida*) or caught after landing (*P. flavocincta*) and released at the end of the respective 5-min test phase. Workers were allowed

to participate in all three trials. To avoid pseudo-replication (*A. mellifera*, *M. subnitida*), only the first landing of an individual in each test phase was considered for the analysis. During the third test, all foragers were captured and killed by freezing to avoid pseudo-replication.

Testing the Impact of Color

In the second experimental series, we investigated the impact of color on the choice of food sites by workers. In this experimental series, we performed two different trial series with each bee species. Each trial consisted of two sets of a 30-min training phase and a subsequent 5-min test phase, switching the feeder positions in pseudo-randomized order (**Supplementary Table S1**). After the training phase (training feeder either blue or yellow; **Supplementary Table S1**), the training feeder was removed, and we offered the incoming bees a blue- and a yellow-colored feeder during the test phase, one at each feeding site (**Supplementary Table S1**). In trial series 1 (C1–C4; **Supplementary Table S1**), bees were trained to blue feeders in the first three training phases and a yellow feeder in the fourth (training to blue, retraining to yellow). In trial series 2 (C5–C8; **Supplementary Table S1**), foragers were trained to yellow feeders during three training phases and a blue feeder in the last training phase (training to yellow, retraining to blue). For the test phases, we used alcohol-cleaned feeders to eliminate the influence by any potential scent marks. During the test phase, both feeders offered sugar solution (50% weight on weight). Each trial series was repeated three to five times with different individuals. The bee species (*A. mellifera*, *M. subnitida*, and *P. flavocincta*) were tested separately and workers that visited the feeder were either marked with nail polish (*A. mellifera* and *M. subnitida*) or caught after landing on a feeder (*P. flavocincta*) and released at the end of the respective 5-min test phase. To avoid pseudo-replication (*A. mellifera*, *M. subnitida*), only the first landing of an individual in each test was considered for the analysis. During the fourth test, all workers were captured and killed by freezing.

Testing the Impact of Location

To test whether bees visited the feeding site closer to the nest (site 1, 15 m) more often than the farther feeding site (site 2, 17 m) the results of all above described tests (scent marks and color) were analyzed concerning the influence of distance.

Statistics

The statistical program R was used to analyze the data (R Development Core Team, 2019). The data were analyzed by testing the bees' choices (the first decision of each test) for the different parameters (scent marks, color, distance) using a generalized linear mixed model (GLMM). We used the "lme4" package of R to analyze choices of the bees, which were assessed using GLMM with Poisson distribution of data (Bates et al., 2009; R Development Core Team, 2019). We analyzed the number of choices for each test as fixed effect and the position of the stimuli were used as random effect of the model when testing the influence of color and scent marks, while these parameters were used as random effect when testing the impact of distance on the bees' choice behavior.

RESULTS

In the first experimental series (influence of scent marks), foragers of all three bee species significantly preferred the previously visited training feeder over the clean feeder (**Figure 1**; *M. subnitida*: $n = 239$, z-value $= -8.346$, $p < 0.001$; *P. flavocincta*: $n = 355$, z-value $= -12.15$, $p < 0.001$; *A. mellifera*: $n = 303$, z-value $= -10.46$, $p < 0.001$).

In the second experimental series, we investigated the influence of color on the feeder choice by the three bee species. After training to a blue-colored feeder, all three species significantly preferred the blue feeder over the yellow feeder (**Figure 2A**; *M. subnitida*: $n = 250$, z-value $= -10.24$, $p < 0.001$; *P. flavocincta*: $n = 230$, z-value $= -8.821$, $p < 0.001$; *A. mellifera*: $n = 538$, z-value $= -10.85$, $p < 0.001$). When these workers were retrained to forage on a yellow feeder during the last training phase, the two stingless bee species significantly preferred the yellow feeder while honeybee workers visited both colors equally (**Figure 2B**; *M. subnitida*: $n = 124$, z-value $= 2.667$, $p = 0.007$; *P. flavocincta*: $n = 71$, z-value $= 3.756$, $p < 0.001$; *A. mellifera*: $n = 278$, z-value $= 0.6$, $p = 0.549$). When workers were initially trained to a yellow-colored feeder, both stingless bee species preferred the yellow feeder significantly over the blue feeder during the test, while *A. mellifera* preferred the blue feeder (**Figure 2C**; *M. subnitida*: $n = 199$, z-value $= 3.318$ $p < 0.001$; *P. flavocincta*: $n = 303$, z-value $= 3.141$, $p = 0.002$; *A. mellifera*: $n = 556$, z-value $= 5.863$, $p < 0.001$). Retraining to a blue feeder in the last training phase lead to a significant preference of the blue colored feeder in all three species (**Figure 2D**; *M. subnitida*: $n = 52$, z-value $= -2.95$, $p = 0.003$; *P. flavocincta*: $n = 61$, z-value $= -2.632$, $p = 0.008$; *A. mellifera*: $n = 213$, z-value $= -6.74$, $p < 0.001$).

When analyzing the influence of the feeders' positions on the food source choice, we observed that *M. subnitida* visited the feeding site closer to the nest during the scent mark trials (site 1, 15 m) significantly more often than the farther site (site 2, 17 m) (**Figure 3**; $n = 239$, z-value $= -8.467$, $p < 0.001$), while

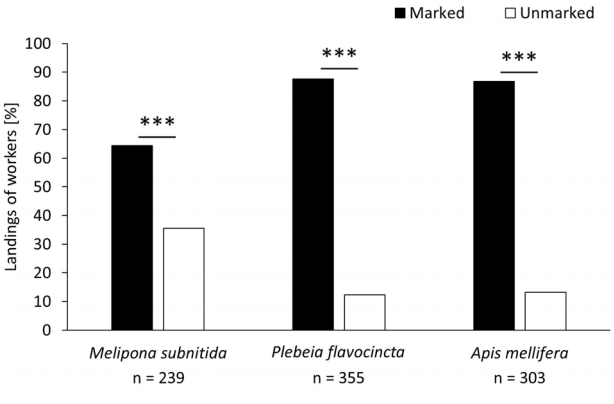


FIGURE 1 | Landings of workers on a scent-marked and an unmarked feeder. A generalized linear mixed model was used for statistical analysis (***$p < 0.001$).

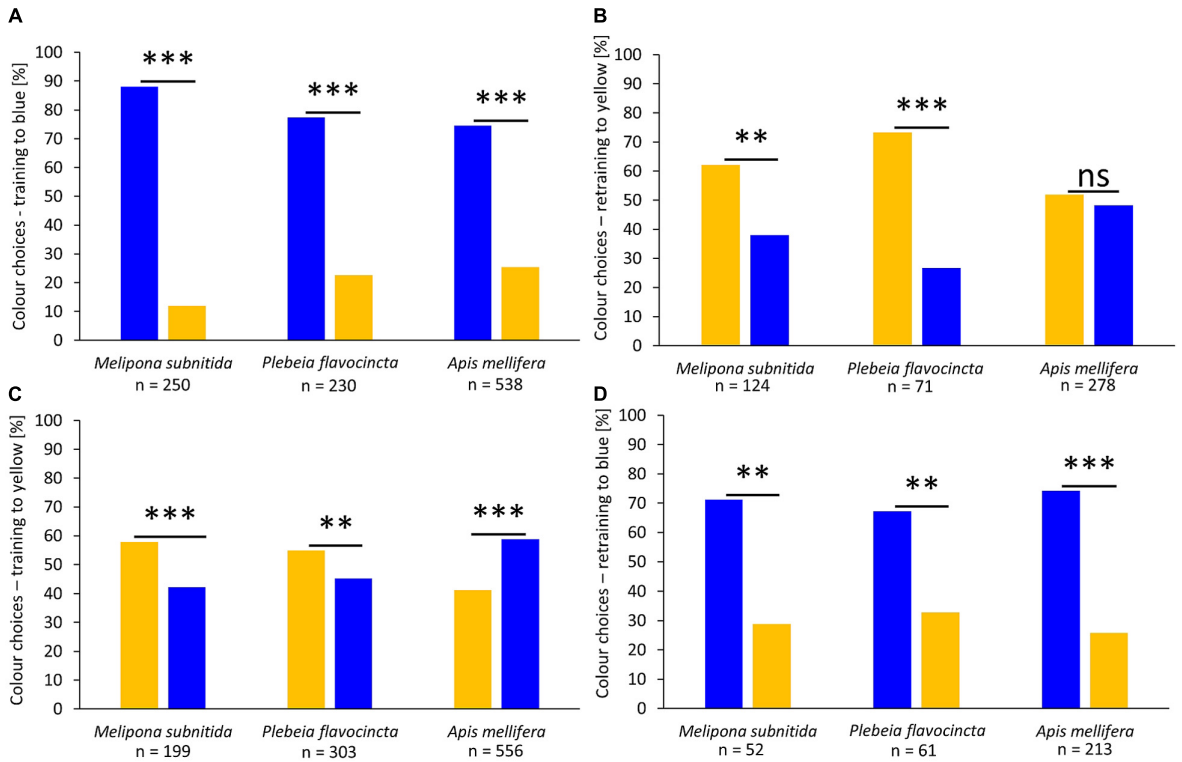


FIGURE 2 | Color choices after training sessions. The three tested bee species were trained to forage on either a blue feeder **(A)** or a yellow feeder **(C)**. Furthermore, the workers were retrained to the opposite color **(B,D)**. A generalized linear mixed model was used for statistical analysis (**$p < 0.01$; ***$p < 0.001$; ns = not significant $p \geq 0.05$).

choosing randomly when tested for color differences (**Figure 3**; blue: $n = 250$, z-value = −0.045, $p = 0.964$; yellow: $n = 199$, z-value = 1.502, $p = 0.133$). Workers of *A. mellifera* significantly preferred the closer feeding site when they were trained to yellow (**Figure 3**; $n = 556$, z-value = −6.147, $p < 0.001$), but did not distinguish between the two sites when trained for blue (**Figure 3**; blue: $n = 538$, z-value = −0.951, $p = 0.342$; scent marks: $n = 199$, z-value = −1.502, $p = 0.133$). In the trial concerning scent marks workers of *A. mellifera* significantly preferred the farther away feeding site (**Figure 3**; $n = 303$, z-value = −10.46, $p < 0.001$). Workers of *P. flavocincta* preferred the closer feeding site when trained to blue or yellow (**Figure 3**; blue: $n = 230$, z-value = −5.036, $p < 0.001$; yellow: $n = 71$, z-value = −4.856, $p < 0.001$) and significantly visited the farther site when tested concerning scent marks (**Figure 3**; $n = 355$, z-value = −2.548, $p = 0.011$).

DISCUSSION

The results of this study show that the response to the color of feeder, scent marks, and locations differs among the tested species *P. flavocincta*, *M. subnitida*, and *A. mellifera*. Our results confirm previous findings about the important role of color for food plant detection in honeybees and add further findings to the diverse and sometimes less important role of color for food plant detection in stingless bees. In previous studies of color preferences in stingless bees, the results varied among species. While three species of the genus *Melipona* chose colors poorly, *Tetragonula carbonaria* chose colors according to their hue and *Partamona helleri* showed similar color choices as *A. mellifera* preferring spectrally purer colors and bluish color hues (Rohde et al., 2013; Dyer et al., 2016; Koethe et al., 2016, 2018). Particularly for the honeybee, it has been shown that besides innate preferences, absolute or differential conditioning and behavioral plasticity play important roles in how they exploit color information (Reser et al., 2012), and that strong color preferences impede learning of other features (Morawetz et al., 2013).

Workers of *A. mellifera* orientated most strongly according to colors. The blue-colored feeder was preferred in all tests with exception of the retraining to yellow, where *A. mellifera* showed no depicted choice for one of the two colors and the two stingless bee species preferred the yellow feeder. This is in accordance to previous studies showing that *A. mellifera* prefers blue colors over other color hues (Giurfa et al., 1995; Horridge, 2007). The two stingless bee species chose feeders according to their colors but rather preferred the feeder color of the previous training. Only when initially trained to yellow they showed weak (*M. subnitida*) or no preferences for the trained color (*P. flavocincta*). This preference for blue is in accordance with previous results of stingless bees, but also suggests that

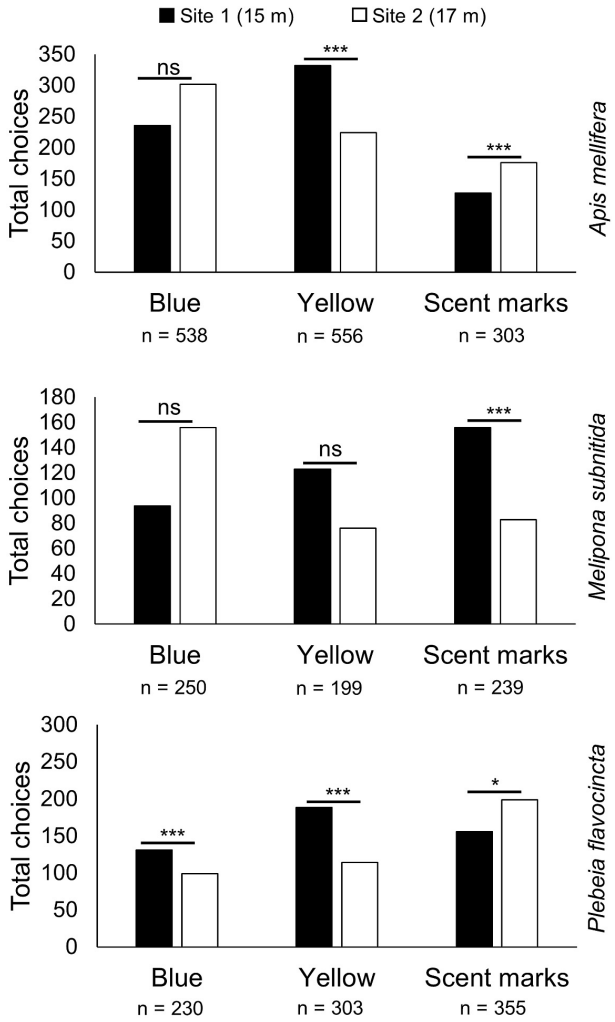


FIGURE 3 | Landings of workers depending on the feeding site. The number of landings at the feeding sites with 15 m (site 1, black) and 17 m (site 2, white) distance to the hive were compared for *A. mellifera*, *M. subnitida*, and *P. flavocincta* for the two color trials, blue and yellow, and the scent mark trial (generalized linear mixed model $^{*}p < 0.05$; $^{***}p < 0.001$; ns = not significant $p \geq 0.05$).

it is weaker in stingless bees than in honeybees (Dyer et al., 2016; Koethe et al., 2016). An explanation for less visually driven behavior in stingless bees could be the size differences compared to honeybees. *P. flavocincta* reaches a body size of 3.6–4.1 mm, *M. subnitida* of 7.5 mm, and *A. mellifera* is the largest of the three species with 13–16 mm (Hrncir and Maia-Silva, 2013; Maia-Silva et al., 2015; Imperatriz-Fonseca et al., 2017). Especially the size of the eyes, which is associated with body size, can impact the visual capacities of bees (Streinzer et al., 2016). Workers of *P. flavocincta* are rather small; consequently, their eyes are also small leading to poorer visual capabilities.

Both stingless bees and honeybees use scent cues to evaluate reward availability of food resources (Nunez, 1967; Butler et al., 1969; Ferguson and Free, 1979; Free and Williams, 1983; Corbet et al., 1984; Giurfa and Nunez, 1992; Giurfa, 1993; Stout et al., 1998; Williams, 1998; Stout and Goulson, 2001). In this study, all three species showed preferences for the marked feeder over the unmarked one. *P. flavocincta* and *A. mellifera* chose the marked feeder consistently (∼88% of choices), while *M. subnitida* preferred the marked feeder, but visited it less frequently (∼64% of choices).

Plebeia flavocincta was the only species that significantly preferred the closer feeding site when tested concerning colors and the farther feeding site when tested regarding scent marks. One interpretation is that *P. flavocincta* does not differentiate between colors and choses the closer feeding site, while the preference during the scent mark trial could be based on the fact that the scent marked feeder was positioned twice at the farther site and only once at the closer site. In contrast, *M. subnitida* was the only species in the scent mark trials that visited the food site with shorter distance to the hive more frequently. It seems likely that *M. subnitida* orientates on location rather than on scent marks. Previous studies showed that species of the genus *Melipona* mark food sites directly and do not lay scent trails (Hrncir et al., 2004). In order to recruit new foragers, it seems possible that *M. subnitida* relies strongly on piloting—leading new foragers from hive to food site during flight (Nieh et al., 2003). Foragers of *M. subnitida* could be observed to frequently arrive in small groups, while *A. mellifera* and *P. flavocincta* workers seemed more independent from each other. Scent marks play an important role for the communication of reward availability, but their impact on recruitment seems dependent on the specific strategy used by species (Free and Williams, 1983; Corbet et al., 1984; Giurfa and Nunez, 1992; Giurfa, 1993; Stout et al., 1998; Stout and Goulson, 2001; Schmidt et al., 2003). The attractiveness of scent marks, whether or not they were used for recruitment purposes, appears to be strong because scent-marked feeders were preferred by all three tested bee species. During the experiments workers foraged in groups and could be influenced by the presence of other individuals. An influence by social facilitation (Wilson, 1971) could not be excluded during the experiments, but when comparing the results for choices of blue and yellow feeders, after the respective training, an influence solely by the presence of conspecifics seems unlikely.

Another aspect that can explain the diverse results for the three tested bee species could be their natural habitat. *M. subnitida* originates from the Caatinga, which is an open habitat, while *P. flavocincta* inhabits a spacious habitat that extends from the Caatinga to the Atlantic Rainforest, which is a densely vegetated forest (Imperatriz-Fonseca et al., 2017). Because of its domestication, the honeybee is widespread all over the world. It originates from diverse habitats of Europe, the Middle East, and Africa. Open habitats are brightly illuminated, while forest habitats are characterized by dim-light conditions (Endler, 1992). Based on the light conditions of their respective habitat, it appears to be possible that *M. subnitida* and *A. mellifera* could rely to a greater extent on visual signals than *P. flavocincta* that encounters dim-light conditions and a less visually structured vegetation. In a densely vegetated habitat, scent marks could be a more reliable signal to guide foragers to a food source. Furthermore, temperate and sub-tropical regions experience

more distinct seasons concerning weather conditions and the rhythm of flowering plants is directly influenced, while tropical and semi-arid regions have more steady weather conditions but are challenging for their inhabitants because of high temperatures (Prado, 2003; Zanella and Martins, 2003; Machado and Lopes, 2004; Maia-Silva et al., 2012, 2015; Hrncir et al., 2019).

Social bee species that face seasonal variations mass-collect floral resources for provision of the hive (Ramalho, 2004). These variations in floral resource availability could be another explanation for more distinct preferences for visual signals in honeybees when compared to tropical species, like *M. subnitida* and *P. flavocincta*, because only honeybees face strong seasonal variations (Michener, 1974; Kleinert-Giovannini, 1982; Roubik, 1982a; Seeley, 1985). Nonetheless, this would not explain the differences between *M. subnitida* and *P. flavocincta*.

CONCLUSION

The three tested bee species reacted vaguely similar to color, scent marks, and location of food sources, but their main focus varies: While *A. mellifera* choose food sites according to both color and scent marks, *M. subnitida* orientates on location and color of food sites, and *P. flavocincta* relies mainly on scent marks. These variations are possibly based on different recruitment mechanisms (e.g., waggle dance of honeybees vs. piloting, excited movements, vibration, and scent mark deposition by stingless bees) or they could be the result of adaptations to the bees' respective habitat and obliged morphological constraints. Although highly eusocial stingless and honeybees do not communicate the color of flowers to nestmates (Michelsen, 2014), flower color has a large impact on foraging decisions. This impact is demonstrated by the results of this study, that bees exhibit a spontaneous response to color cues and that they memorize color cues following experience; spontaneous response of bees and discrimination after conditioning might rely on different color parameters, such as color saturation and color hue (Rohde et al., 2013). Flower color has also been identified as a floral filter excluding bees from visiting the less preferred flower colors, i.e., red, UV-absorbing and white, UV-reflecting hummingbird-pollinated flowers (Lunau et al., 2011). Stingless bees are known as nectar robbers of hummingbird-pollinated flowers (Roubik, 1982b); it remains to be tested if the less pronounced color preferences in stingless bees are helpful for finding food on flowers displaying colors that are not adapted to bee-color vision and color preferences.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KL conceived and developed the research concept and supervised the study. SK designed the experiments. MH and KL supported the design of the experiments. SK, VF, LR, and SB conducted the experiments and collected and analyzed the data. SK wrote the manuscript. SB, VF, LR, SB, MH, and KL supported the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00516/full#supplementary-material

FIGURE S1 | Spectral reflectance curves of colored feeders.

TABLE S1 | Position of feeders in training and test phase. The order of tests was pseudo-randomized to ensure no influence of test order on the decisions of workers. Two experimental trials were conducted comprising four choice experiments each. C1–C4 are the tests which focused on blue color, while the tests C5–C8 focused on yellow color. SM1–3 = are tests which analyzed the impact of scent marks; m = marked feeder; u = unmarked feeder; C1–8 = are the tests analyzing the impact of color; b = blue; y = yellow; site 1 = 15 m distance to the hive; site 2 = 17 m distance to the hive.

REFERENCES

Ackerman, J. D., and Montalvo, A. M. (1985). Longevity of euglossine bees. *Biotropica* 17, 79–81.

Aguilar, I., Fonseca, A., and Biesmeijer, J. C. (2005). Recruitment and communication of food source location in three species of stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie* 36, 313–324. doi: 10.1051/apido:2005005

Amiet, F., and Krebs, A. (2012). *Bienen Mitteleuropas: Gattungen, Lebensweise, Beobachtung*. Bern: Haupt.

Barth, F. G., Hrncir, M., and Jarau, S. (2008). Signals and cues in the recruitment behavior of stingless bees (Meliponini). *J. Comp. Physiol. A* 194, 313–327. doi: 10.1007/s00359-008-0321-7

Bates, D., Maechler, M., and Dai, B. (2009). *lme4: Linear Mixed-Effects Models Using S4 Classes. R package version 0.999375–999331*.

Biesmeijer, J. C., and Slaa, E. J. (2004). Information flow and organization of stingless bee foraging. *Apidologie* 35, 143–157. doi: 10.1051/apido:2004003

Biesmeijer, J. C., and Slaa, J. E. (2006). The structure of eusocial bee assemblages in Brazil. *Apidologie* 37, 240–258. doi: 10.1051/apido:2006014

Biesmeijer, J. C., and Toth, E. (1998). Individual foraging, activity level and longevity in the stingless bee *Melipona beecheii* in Costa Rica (Hymenoptera, Apidae, Meliponinae). *Insectes Soc.* 45, 427–443. doi: 10.1007/s000400050099

Breed, M. D., Stocker, E. M., Baumgartner, L. K., and Vargas, S. A. (2002). Time-place learning and the ecology of recruitment in a stingless bee, *Trigona amalthea* (Hymenoptera, Apidae). *Apidologie* 33, 251–258. doi: 10.1051/apido:2002018

Butler, C. G., Fletcher, D. J. C., and Watler, D. (1969). Nest-entrance marking with pheromones by the honeybee, *Apis mellifera* L., and by the wasp, *Vespula vulgaris* L. *Anim. Behav.* 17, 142–147. doi: 10.1016/0003-3472(69)90122-5

Cane, J. H., and Snipes, S. (2006). "Characterizing floral specialization by bees: analytical methods and a revised lexicon for oligolecty," in *Plant-Pollinator Interactions*, eds N. M. Waser and J. Ollerton (Chicago, IL: University Press of Chicago), 99–122.

Cartwright, B. A., and Collett, T. S. (1983). Landmark learning in bees. *J. Comp. Physiol. A* 151, 521–543. doi: 10.1007/bf00605469

Cheng, K., Collett, T. S., Pickhard, A., and Wehner, R. (1987). The use of visual landmarks by honeybees: bees weight landmarks according to their distance from the goal. *J. Comp. Physiol. A* 161, 469–475. doi: 10.1007/bf00603972

Cheng, K., Collett, T. S., and Wehner, R. (1986). Honeybees learn the colours of landmarks. *J. Comp. Physiol. A* 159, 69–73. doi: 10.1007/bf00612497

Chittka, L., Geiger, K., and Kunze, J. (1995). The influences of landmarks on distance estimation of honey bees. *Anim. Behav.* 50, 23–31. doi: 10.1006/anbe.1995.0217

Cockerell, T. D. A. (1912). New bees from Brazil. *Psyche* 19, 41–61. doi: 10.1155/1912/25740

Corbet, S. A., Kerslake, C. J. C., Brown, D., and Morland, N. E. (1984). Can bees select nectar-rich flowers in a patch? *J. Apic. Res.* 23, 234–242. doi: 10.1080/00218839.1984.11100638

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016). Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, F. C. (2002). The biology of the dance language. *Annu. Rev. Entomol.* 47, 917–949. doi: 10.1146/annurev.ento.47.091201.145306

Eltz, T. (2006). Tracing pollinator footprints on natural flowers. *J. Chem. Ecol.* 32, 907–915. doi: 10.1007/s10886-006-9055-6

Endler, J. A. (1992). The colour of light in forests and its implications. *Ecol. Monogr.* 63, 1–27. doi: 10.2307/2937121

Engels, W., and Imperatriz-Fonseca, V. L. (1990). "Caste development, reproductive strategies, and control of fertility in honey bees and stingless bees," in *Social Insects: An Evolutionary Approach to Castes and Reproduction*, ed. W. Engels (Berlin: Springer), 167–230. doi: 10.1007/978-3-642-74490-7_9

Esch, H. (1961). Über die Schallerzeugung beim Werbetanz der Honigbiene. *Z. Vgl. Physiol.* 45, 1–11. doi: 10.1007/bf00297754

Esch, H., Esch, I., and Kerr, W. E. (1965). Sound: an element common to communication of stingless bees and honey bees. *Science* 149, 320–321. doi: 10.1126/science.149.3681.320

Farina, W. M., Grüter, C., Acosta, L., and Mc Cabe, S. (2007). Honeybees learn floral odors while receiving nectar from foragers within the hive. *Naturwissenschaften* 94, 55–60. doi: 10.1007/s00114-006-0157-3

Farina, W. M., Grüter, C., and Díaz, P. C. (2005). Social learning of floral odours inside the honeybee hive. *Proc. Biol. Sci.* 272, 1923–1928. doi: 10.1098/rspb.2005.3172

Ferguson, A. W., and Free, J. B. (1979). Production of a forage-marking pheromone by the honeybee. *J. Apic. Res.* 18, 128–135. doi: 10.1080/00218839.1979.11099956

Free, J. B. (1963). The flower constancy of honeybees. *J. Anim. Ecol.* 32, 119–131.

Free, J. B., and Williams, I. H. (1983). Foraging behaviour of honeybees and bumble bees on Brussels sprout grown to produce hybrid seed. *J. Apic. Res.* 22, 94–97. doi: 10.1080/00218839.1983.11100566

Giurfa, M. (1993). The repellent scent-mark of the honeybee *Apis mellifera ligustica* and its role as communication cue during foraging. *Insectes Soc.* 40, 59–67. doi: 10.1007/bf01338832

Giurfa, M., Nunez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comp. Physiol. A* 177, 247–259.

Giurfa, M., and Nunez, J. A. (1992). Honeybees mark with scent and reject recently visited flowers. *Oecologia* 89, 113–117. doi: 10.1007/BF00319022

Goulson, D., Hawson, S. A., and Stout, J. C. (1998). Foraging bumblebees avoid flowers already visited by conspecifics or by other bumblebee species. *Anim. Behav.* 55, 199–206. doi: 10.1006/anbe.1997.0570

Gumbert, A. (2000). Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48, 36–43. doi: 10.1007/s002650000213

Han, F., Wallberg, A., and Webster, M. T. (2012). From where did the Western honeybee (*Apis mellifera*) originate? *Ecol. Evol.* 2, 1949–1957. doi: 10.1002/ece3.312

Horridge, A. (2007). The preferences of the honeybee (*Apis mellifera*) for different visual cues during the learning process. *J. Insect Physiol.* 53, 877–889. doi: 10.1016/j.jinsphys.2006.12.002

Howard, S. R., Shrestha, M., Schramme, J., Garcia, J. E., Avarguès-Weber, A., Greentree, A. D., et al. (2019). Honeybees prefer novel insect-pollinated flower shapes over bird-pollinated flower shapes. *Curr. Zool.* 65, 457–465. doi: 10.1093/cz/zoy095

Hrncir, M., and Barth, F. G. (2014). "Vibratory communication in stingless bees (Meliponini): the challenge of interpreting the signals," in *Studying Vibrational Communication*, eds R. B. Cocroft, M. Gogala, P. S. M. Hill, and A. Wessel (Berlin: Springer), 349–374. doi: 10.1007/978-3-662-43607-3_18

Hrncir, M., Jarau, S., Zucchi, R., and Barth, F. G. (2004). On the origin and properties of scent marks deposited at the food source by a stingless bee, *Melipona seminigra*. *Apidologie* 35, 3–13. doi: 10.1051/apido:2003069

Hrncir, M., and Maia-Silva, C. (2013). "On the diversity of foraging-related traits in stingless bees," in *Pot-Honey: A Legacy of Stingless Bees*, eds P. Vit, S. R. M. Pedro, and D. Roubik (New York, NY: Springer), 201–215. doi: 10.1007/978-1-4614-4960-7_13

Hrncir, M., Maia-Silva, C., Mc Cabe, S. I., and Farina, W. M. (2011). The recruiter's excitement – features of thoracic vibrations during the honey bee's waggle dance related to food source profitability. *J. Exp. Biol.* 214, 4055–4064. doi: 10.1242/jeb.063149

Hrncir, M., Maia-Silva, C., Teixeira-Souza, V. H. S., and Imperatriz-Fonseca, V. L. (2019). Stingless bees and their adaptations to extreme environments. *J. Comp. Physiol. A* 205, 415–426. doi: 10.1007/s00359-019-01327-3

Imperatriz-Fonseca, V. L., Koedam, D., and Hrncir, M. (2017). *A Abelha Jandaíra no Passado, No Presente e No Futuro, EdUFERSA*. Mossóro: Editora Universitária.

Jarau, S. (2009). "Chemical communication during food exploitation in stingless bees," in *Food Exploitation by Social Insects: Ecological, Behavioral, and Theoretical Approaches*, eds S. Jarau and M. Hrncir (Boca Raton, FL: CRC Press), 223–249. doi: 10.1201/9781420075618.ch12

Jarau, S., Hrncir, M., Ayasse, M., Schulz, C., Francke, W., Zucchi, R., et al. (2004). A stingless bee (*Melipona seminigra*) marks food sources with a pheromone from its claw retractor tendons. *J. Chem. Ecol.* 30, 793–804. doi: 10.1023/b:joec.0000028432.29759.ed

Jesus, T. N. C. S., Venturieri, G. C., and Contrera, F. A. L. (2014). Time-place learning in the bee *Melipona fasciculata* (Apidae, Meliponini). *Apidologie* 45, 257–265. doi: 10.1007/s13592-013-0245-2

Johnson, L. K. (1983). "Foraging strategies and the structure of stingless bee communities in Costa Rica," in *Social Insects in the Tropics 2*, ed. P. Jaisson (Paris: Universite Paris-Nord), 31–58.

Kleinert-Giovannini, A. (1982). The influence of climatic factors on flight activity of *Plebeia emerina* Friese (Hymenoptera, Apidae, Meliponinae) in winter. *Rev. Bras. Entomol.* 26, 1–13.

Koethe, S., Banysch, S., Alves-dos-Santos, I., and Lunau, K. (2018). Spectral purity, intensity and dominant wavelength: disparate colour preferences of two Brazilian stingless bee species. *PLoS One* 13:e0204663. doi: 10.1371/journal.pone.0204663

Koethe, S., Bossems, J., Dyer, A. G., and Lunau, K. (2016). Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*. *J. Comp. Physiol. A* 202, 615–627. doi: 10.1007/s00359-016-1115-y

Köthe, S. (2019). *Impact of Colour Preferences on the Foraging Behaviour of Tropical Stingless Bees*. Doctoral dissertation, Heinrich-Heine-Universität Düsseldorf, Düsseldorf.

Lehrer, M., Horridge, G. A., Zhang, S. W., and Gadagkar, R. (1995). Shape and vision in bees: innate preference for flower-like patterns. *Philos. Trans. R. Soc. B* 347, 123–137. doi: 10.1098/rstb.1995.0017

Lindauer, M., and Kerr, W. E. (1958). Die gegenseitige Verständigung bei den stachellosen Bienen. *Z. Vgl. Physiol.* 41, 405–434. doi: 10.1007/bf00344263

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: flower dummy experiments with bumblebees. *J. Comp. Physiol. A* 166, 827–834.

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19.

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 178, 477–489.

Machado, I. C., and Lopes, A. V. (2004). Floral traits and pollination systems in the Caatinga, a Brazilian tropical dry forest. *Ann. Bot.* 94, 365–376. doi: 10.1093/aob/mch152

Maia-Silva, C., Da Silva, C. I., Hrncir, M., de Queiroz, R. T., and Imperatriz-Fonseca, V. L. (2012). *Guia de Plantas Visitadas por Abelhas na Caatinga*. Fortaleza: Editora Fundação Brasil Cidadão.

Maia-Silva, C., Hrncir, M., Da Silva, C. I., and Imperatriz-Fonseca, V. L. (2015). Survival strategies of stingless bees (*Melipona subnitida*) in an unpredictable environment, the Brazilian tropical dry forest. *Apidologie* 46, 631–643. doi: 10.1007/s13592-015-0354-1

Mc Cabe, S. I., and Farina, W. M. (2009). Odor information transfer in the stingless bee *Melipona quadrifasciata*: effect of in-hive experiences on classical conditioning of proboscis extension. *J. Comp. Physiol. A* 195, 113–122. doi: 10.1007/s00359-008-0391-6

Mc Cabe, S. I., Hartfelder, K., Santana, W. C., and Farina, W. M. (2007). Odor discrimination in classical conditioning of proboscis extension in two stingless bee species in comparison to Africanized honeybees. *J. Comp. Physiol. A* 193, 1089–1099. doi: 10.1007/s00359-007-0260-8

Menzel, R. (1967). Untersuchungen zum erlernen von spektralfarben durch die Honigbiene (*Apis mellifica*). *Z. Vgl. Physiol.* 56, 22–62. doi: 10.1007/bf00333562

Menzel, R., Greggers, U., Smith, A., Berger, S., Brandt, R., Brunke, S., et al. (2005). Honey bees navigate according to a map-like spatial memory. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3040–3045. doi: 10.1073/pnas.0408550102

Michelsen, A. (2014). "Mechanical signals in honeybee communication," in *Studying Vibrational Communication. Animal Signals and Communication*, Vol. 3, eds R. Cocroft, M. Gogala, P. Hill, and A. Wessel (Berlin: Springer), 333–347. doi: 10.1007/978-3-662-43607-3_17

Michener, C. D. (2013). "The Meliponini," in *Pot-Honey: A Legacy of Stingless Bees*, eds P. Vit, S. R. M. Pedro, and D. Roubik (New York, NY: Springer), 3–17. doi: 10.1007/978-1-4614-4960-7_1

Michener, D. (1974). *The Social Behaviour of the Bees*. Cambridge, MA: Harvard University Press.

Morawetz, L., Svoboda, A., Spaethe, J., and Dyer, A. G. (2013). Blue colour preference in honeybees distracts visual attention for learning closed shapes. *J. Comp. Physiol. A* 199, 817–827. doi: 10.1007/s00359-013-0843-5

Nieh, J. C., Contrera, F. A., Ramírez, S., and Imperatriz-Fonseca, V. L. (2003). Variation in the ability to communicate three-dimensional resource location by stingless bees from different habitats. *Anim. Behav.* 66, 1129–1139. doi: 10.1006/anbe.2003.2289

Nieh, J. C., Contrera, F. A. L., Yoon, R. R., Barreto, L. S., and Imperatriz-Fonseca, V. L. (2004). Polarized short odor-trail recruitment communication by a stingless bee, *Trigona spinipes*. *Behav. Ecol. Sociobiol.* 56, 435–448.

Nunez, J. A. (1967). Sammelbienen markieren versiegende Futterquellen durch Duft. *Naturwissenschaften* 54, 322–323. doi: 10.1007/bf00640625

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: how bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Pessotti, I., and Lé'Sénéchal, A. M. (1981). Aprendizagem em abelhas. I. Discriminacao simples em onze especies. *Acta Amazon* 11, 653–658. doi: 10.1590/1809-43921981113653

Prado, D. (2003). "As caatingas da América do Sul," in *Ecologia e Conservação da Caatinga*, eds I. R. Leal, M. Tabarelli, and J. M. C. Silva (Recife: Editora Universitária UFPE), 75–134.

R Development Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raine, N. E., and Chittka, L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Ramalho, M. (2004). Stingless bees and mass flowering trees in the canopy of Atlantic Forest: a tight relationship. *Acta Bot. Brasilica* 18, 37–47. doi: 10.1590/s0102-33062004000100005

Reinhard, J., Srinivasan, M. V., Guez, D., and Zhang, S. W. (2004). Floral scents induce recall of navigational and visual memories in honeybees. *J. Exp. Biol.* 207, 4371–4381. doi: 10.1242/jeb.01306

Reinhard, J., Srinivasan, M. V., and Zhang, S. W. (2006). Complex memories in honeybees: can there be more than two? *J. Comp. Physiol. A* 192, 409–416. doi: 10.1007/s00359-005-0079-0

Reser, D. H., Wijesekara Witharanage, R., Rosa, M. G. P., and Dyer, A. G. (2012). Honeybees (*Apis mellifera*) learn color discriminations via differential conditioning independent of long wavelength (green) photoreceptor modulation. *PLoS One* 7:e48577. doi: 10.1371/journal.pone.0048577

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Roldão-Sbordoni, Y. S., Nascimento, F. S., and Mateus, S. (2018). Estimating colonies of *Plebeia droryana* (Friese, 1900) (Hymenoptera, Apidae, Meliponini): adults, brood and nest structure. *Sociobiology* 65, 280–284.

Roselino, A. C., Rodrigues, A. V., and Hrncir, M. (2016). Stingless bees (*Melipona scutellaris*) learn to associate footprint cues at food sources with a specific reward context. *J. Comp. Physiol. A* 202, 657–666. doi: 10.1007/s00359-016-1104-1

Roubik, D. W. (1982a). Seasonality in colony food storage, brood production and adult survivorship: studies of Melipona in tropical forest (Hymenoptera: Apidae). *J. Kansas Entomol. Soc.* 55, 789–800.

Roubik, D. W. (1982b). The ecological impact of nectar-robbing bees and pollinating hummingbirds on a Tropical Shrub. *Ecology* 63, 354–360. doi: 10.2307/1938953

Roubik, D. W. (2006). Stingless bee nesting biology. *Apidologie* 37, 124–143. doi: 10.1051/apido:2006026

Roubik, D. W., and Buchmann, S. L. (1984). Nectar selection by Melipona and *Apis mellifera* (Hymenoptera: Apidae) and the ecology of nectar intake by bee colonies in a tropical forest. *Oecologia* 61, 1–10. doi: 10.1007/BF00379082

Saleh, N., and Chittka, L. (2006). The importance of experience in the interpretation of conspecific chemical signals. *Behav. Ecol. Sociobiol.* 61, 215–220. doi: 10.1007/s00265-006-0252-7

Sánchez, D., Nieh, J. C., and Vandame, R. (2008). Experience-based interpretation of visual and chemical information at food sources in the stingless bee *Scaptotrigona mexicana*. *Anim. Behav.* 76, 407–414. doi: 10.1016/j.anbehav.2008.04.003

Schmidt, V. M., Zucchi, R., and Barth, F. G. (2003). A stingless bee marks the feeding site in addition to the scent path (*Scaptotrigona depilis*). *Apidologie* 32, 237–248. doi: 10.1051/apido:2003021

Schmidt, V. M., Zucchi, R., and Barth, F. G. (2005). Scent marks left by *Nannotrigona testaceicornis* at the feeding site: cues rather than signals. *Apidologie* 36, 285–291. doi: 10.1051/apido:2005002

Schwarz, H. F. (1932). The genus Melipona. The type genus of the Meliponidae or stingless bees. *Bull. Am. Mus. Nat. Hist.* 63, 231–460.

Seeley, T. D. (1985). *Honeybee Ecology: A Study of Adaptation in Social Life*. Princeton, NJ: Princeton University Press.

Slaa, E. J., and Hughes, W. H. O. (2009). "Local enhancement, local inhibition, eavesdropping, and the parasitism of social insect communication," in *Food Exploitation by Social Insects: Ecological, Behavioral, and Theoretical Approaches*, eds S. Jarau and M. Hrncir (Boca Raton, FL: CRC Press), 147–164. doi: 10.1201/9781420075618.ch8

Slaa, J. E., Cevaal, A., and Sommeijer, M. J. (1998). Floral constancy in Trigona stingless bees foraging on artificial flower patches: a comparative study. *J. Apic. Res.* 37, 191–198. doi: 10.1080/00218839.1998.11100971

Slaa, J. E., Wassenberg, J., and Biesmeijer, J. C. (2003). The use of field-based social information on eusocial foragers: local enhancement among nestmates and heterospecifics in stingless bees. *Ecol. Entomol.* 28, 369–379. doi: 10.1046/j.1365-2311.2003.00512.x

Spaethe, J., Streinzer, M., Eckert, J., May, S., and Dyer, A. G. (2014). Behavioural evidence of colour vision in free flying stingless bees. *J. Comp. Physiol. A* 200, 485–496. doi: 10.1007/s00359-014-0886-2

Stout, J. C., and Goulson, D. (2001). The use of conspecific and interspecific scent marks by foraging bumblebees and honeybees. *Anim. Behav.* 62, 183–189. doi: 10.1006/anbe.2001.1729

Stout, J. C., Goulson, D., and Allen, J. A. (1998). Repellent scent-marking of flowers by a guild of foraging bumblebees (*Bombus* spp.). *Behav. Ecol. Sociobiol.* 43, 317–326. doi: 10.1007/s002650050497

Streinzer, M., Huber, W., and Spaethe, J. (2016). Body size limits dim-light foraging activity in stingless bees (Apidae: Meliponini). *J. Comp. Physiol. A* 202, 643–655. doi: 10.1007/s00359-016-1118-8

Von Frisch, K. (1967). *The Dance Language and Orientation of Bees*. Cambridge, MA: Harvard University Press.

Waddington, K. D., and Kirchner, W. H. (1992). Acoustical and behavioral correlates of profitability of food sources in honey bee round dances. *Ethology* 92, 1–6. doi: 10.1111/j.1439-0310.1992.tb00945.x

Wille, A. (1983). Biology of the stingless bees. *Annu. Rev. Entomol.* 28, 41–64.

Williams, C. S. (1998). The identity of the previous visitor influences flower rejection by nectar collecting bees. *Anim. Behav.* 56, 673–681. doi: 10.1006/anbe.1998.0794

Wilson, E. O. (1971). *The Insect Societies*. Cambridge, MA: Harvard University Press.

Witjes, S., Witsch, K., and Eltz, T. (2011). Reconstructing the pollinator community and predicting seed set from hydrocarbon footprints on flowers. *Oecologia* 165, 1017–1029. doi: 10.1007/s00442-010-1816-9

Yokoi, T., Goulson, D., and Fujisaki, K. (2007). The use of heterospecific scent marks by the sweat bee *Halictus aerarius*. *Naturwissenschaften* 94, 1021–1024. doi: 10.1007/s00114-007-0285-4

Zanella, F. C. (2000). The bees of the Caatinga (Hymenoptera, Apoidea, Apiformes): a species list and comparative notes regarding their distribution. *Apidologie* 31, 579–592. doi: 10.1051/apido:2000148

Zanella, F. C. V., and Martins, C. F. (2003). "Abelhas da Caatinga: biogeografia, ecologia e conservação," in *Ecologia e Conservação da Caatinga*, eds I. R. Leal, M. Tabarelli, and J. M. C. Silva (Recife: Editora Universitária UFPE), 75–134.

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frontiers in Plant Science

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Climate Predicts UV Floral Pattern Size, Anthocyanin Concentration, and Pollen Performance in *Clarkia unguiculata*

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Given that flower size and pigmentation can mediate plant–pollinator interactions, many studies have focused on pollinator-driven selection on these floral traits. However, abiotic factors such as precipitation, temperature, and solar radiation also contribute to geographic variation in floral color, pattern, and size within multiple species. Several studies have described an ecogeographic pattern within species in which high temperature, high ultraviolet (UV) radiation, low precipitation and/or low latitudes are associated with increased floral anthocyanin production, smaller flowers, and/or larger UV-absorbing floral patterns (nectar guides or bullseyes). However, latitude or elevation is often used as a proxy variable to study variation in floral traits associated with a wide range of climatic variables, making the proximate abiotic drivers of variation difficult to identify. In this study, we tested and corroborated several predictions for how the abiotic environment may directly or indirectly shape geographic patterns of floral color, pattern, and size in *Clarkia unguiculata* (Onagraceae). This study provides the first report of geographic variation in multispectral floral color and pattern in *C. unguiculata*, while also providing an experimental test of the putative protective role of UV absorption for pollen performance. We quantified geographic variation among greenhouse-raised populations in UV floral pattern size, mean UV petal reflectance, anthocyanin concentration, and petal area in *C. unguiculata* across its natural range in California and, using 30 year climate normals for each population, we identified climatic and topographic attributes that are correlated with our focal floral traits. In addition, we examined pollen performance under high and low UV light conditions to detect the protective function (if any) of UV floral patterns in this species. Contrary to our expectations, the nectar guide and the proportion of the petal occupied by the UV nectar guide were largest in low solar UV populations. Estimated floral anthocyanin concentration was highest in populations with high solar UV, which does support our predictions. The size of the UV nectar guide did not affect pollen performance in either of

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the light treatments used in this study. We conclude that, under the conditions examined here, UV-absorbing floral patterns do not serve a direct "pollen protection" function in *C. unguiculata*. Our results only partially align with expected ecogeographic patterns in these floral traits, highlighting the need for research in a wider range of taxa in order to detect and interpret broad scale patterns of floral color variation.

Keywords: geographic variation, flower color, floral evolution, intraspecific variation, pollen performance, UV patterns, nectar guide

INTRODUCTION

Geographic variation in floral color, pattern, and size is well documented in multiple plant species (Streisfeld and Kohn, 2005; Schemske and Bierzychudek, 2007; Arista et al., 2013; Hopkins and Rausher, 2014; del Valle et al., 2015; Sobral et al., 2015; Tripp et al., 2018). Given that flower size and pigmentation can mediate plant–pollinator interactions (Brunet, 2009; Grossenbacher and Stanton, 2014; Koski and Ashman, 2014; Muchhala et al., 2014), many studies have focused on pollinator-driven selection on these floral traits (Rausher, 2008; van der Niet and Johnson, 2012; Grossenbacher and Stanton, 2014; Muchhala et al., 2014). While it is clear that pollinators, and other biotic factors such as florivores, have shaped floral evolution (see van der Niet and Johnson, 2012 for a review), abiotic factors such as UV (ultraviolet) radiation, temperature and precipitation may also drive intraspecific variation in floral color and form (Arista et al., 2013; del Valle et al., 2015; Campbell and Powers, 2015; Koski and Ashman, 2016; Tripp et al., 2018).

Precipitation, temperature, and solar (UV) radiation are associated with variation in floral color within multiple taxa (Schemske and Bierzychudek, 2001 [*Linanthus parryae*]; del Valle et al., 2015 [*Silene littorea*]; Koski and Ashman, 2015 [*Argentina anserina*]). For example, high temperatures and low water availability are sometimes associated with a higher proportion of pink, purple, or blue floral color morphs compared to white ones (Schemske and Bierzychudek, 2001; Berardi, 2014). Anthocyanins (which commonly confer orange, red, blue, or purple coloration to plant tissues) may also provide protection against environmental stressors such as extreme (high or low) temperatures, low water availability, high UV light, or other abiotic factors (Lee and Gould, 2002; Lee and Finn, 2007; Landi et al., 2015). In flower buds, anthocyanins may help to protect developing reproductive tissues from antagonists, while in fully developed flowers, anthocyanins may attract pollinators, and in fruits they may help to attract seed dispersers (Whittall and Strauss, 2006; Landi et al., 2015).

Intraspecific variation in floral and vegetative anthocyanin concentration within and among populations is common and often adaptive (del Valle et al., 2015; Berardi et al., 2016; Menzies et al., 2016). Some studies have shown that populations at low latitudes or in xeric environments may be characterized by small flower size and/or large UV absorbing floral patterns (Herrera, 2005; Lacey et al., 2010; Campbell and Powers, 2015; Koski and Ashman, 2015).

A decade of work suggests that climatic (and topographic) features may be drivers of intraspecific variation in floral color, pattern, and size (Schemske and Bierzychudek, 2007; Arista et al., 2013; Koski and Ashman, 2015; del Valle et al., 2015; Berardi et al., 2016). Collectively, this work suggests that, when examining geographic variation in these floral traits, we should expect a general pattern in which low latitude, high temperature, high solar UV, and low precipitation environments may select (either directly or indirectly) for increased petal anthocyanin production, larger UV-absorbing floral patterns, lower mean UV petal reflectance and/or smaller flowers. If large UV floral patterns confer a direct fitness advantage to individuals in high-UV environments by protecting developing pollen from abiotic stress, we would expect that under high-UV conditions pollen produced by individuals with larger UV floral patterns would perform better than pollen produced by individuals with smaller UV patterns.

To test these predictions, and to contribute toward an understanding of large-scale geographic patterns in these floral features, we quantified UV mean petal reflectance, the proportion of the petal occupied by the UV-absorbing nectar guide, petal area, and estimated floral anthocyanin concentration in greenhouse-raised populations representing eight wild populations of *Clarkia unguiculata* (Onagraceae) that span a wide latitudinal and climatic gradient (**Supplement 1**). We also tested the ability of high solar UV radiation to exert direct positive selection on UV nectar guide size, which has been demonstrated in only one other species to date (Koski and Ashman, 2015).

This study provides the first report of geographic variation in multi-spectral floral color and pattern in *C. unguiculata*, while also providing an experimental test of the putative protective role of UV absorption for pollen performance. In this study we examine greenhouse-raised populations representing eight wild populations of *C. unguiculata* to address the following questions: (1) Is intraspecific variation in floral color, pattern, and size geographically structured? (2) Which specific components of climate/geography (if any) are good predictors of these traits? (3) Does exposure to supplemental UV light during floral development influence floral color (anthocyanin production)? (4) Does UV nectar guide size serve a protective function? In other words, under high UV growing conditions, are flowers with larger UV nectar guides better able to protect their developing pollen from damaging abiotic stress? (5) Do the geographic patterns observed here reflect

what we would expect based on the patterns described in previous research?

MATERIALS AND METHODS

Study Species

Clarkia unguiculata (Onagraceae) is a bee-pollinated, primarily outcrossing, annual species native to California that exhibits tremendous variation in flower color, pattern, and size (Ivey et al., 2016). *C. unguiculata* is pollinated primarily by a set of solitary bee species (the '*Clarkia*' bees) but are also visited by generalists such as *Bombus* spp. (MacSwain et al., 1973; Moeller and Geber, 2005). The "*Clarkia* bees" and *Bombus* are all in the order Hymenoptera. Hymenopteran insects are trichromats and observations from 43 species of Hymenoptera reveal maximal visual sensitivity at 340 nm (UV), 430 nm (blue), and 535 nm (green) (Chittka et al., 1999; Skorupski and Chittka, 2010; Dyer et al., 2012, 2015; Papiorek et al., 2016). Consequently, flower color in these wavelengths is of particular interest to studies of floral color evolution and geographic variation.

The red-purple color of the flowers of *C. unguiculata* is produced by three major anthocyanins: malvidin, cyanidin and delphinidin (Soltis, 1986). Malvidin 3,5-diglucoside appears to predominantly contribute to red-purple flower color in this species (Bowman, 1987).

The flowers of *C. unguiculata* are hermaphroditic and protandrous (i.e., anther maturity precedes stigma receptivity), and each flower bears two whorls of dimorphic stamens. The inner whorl is smaller, shorter and often white; the outer whorl is several millimeters longer, matures later, and bears anthers that range from dark red to purple (Lewis and Lewis, 1955; Peach and Mazer, 2019). We classified the developmental stages of the flowers of *C. unguiculata* into three distinct phases: male stage 1 (when the short anthers release their pollen), male stage 2 (when the long anthers release their pollen), and female (stage 3, when the stigma becomes receptive). Previous work has shown that floral sex stage is a significant source of variation in multiple floral traits in this species (Peach et al., 2020). We included floral sex stage as an independent variable in our statistical analyses (described below) so that we could control for its effects on floral phenotype when testing for and characterizing variation among populations. See **Supplement 2** for a more detailed justification and description of this categorization.

Greenhouse Study

Seeds from eight wild populations of *C. unguiculata* were collected and cultivated for this study. These eight wild populations span a latitudinal range of 33.46826°- 39.68256°, which includes almost the entire range of the species (*C. unguiculata* is endemic to California which spans a ∼32°-42° latitudinal range). Population locations are reported in **Supplement 1**. In 2015-2016, we sampled seeds from 35 maternal families per population. Seeds were placed in coin envelopes (one maternal family per envelope), which were stored in plastic zip-lock bags with silica desiccant in a dark refrigerator at 5°C until use. In Fall 2016, ten seeds per maternal family (35 maternal families × 8 populations) were germinated in agar in 10cm diameter Petri dishes. Petri dishes holding dormant seeds were placed in a dark refrigerator at 5°C for one week to promote germination. After germination, three seedlings per maternal family were randomly selected and planted individually in cone-tainers (20.32 cm in length × 3.81 cm diameter SC10 cone-tainers,[1] Tangent, OR, United States) filled with a custom soil mixture (5:1:1 Sunshine Grow #4, sand, worm castings, and.14.17 grams Island Seed and Feed fertilizer per 3.62 kilograms of soil[2]). Cone-tainers were placed in racks in the greenhouses at UC Santa Barbara and bottom watered for the duration of the study. Plants were grown under controlled temperature (10–18°C nighttime temperature range and a 13–29°C daytime temperature range), and the temperature was logged hourly so that the statistical models used here (described below) could include temperature as an independent variable. The temperature sensor was suspended from the ceiling of the greenhouse, directly between the two rows of light treatments. It is possible that there were differences in temperature and light environment between the light treatments that were not accounted for directly here. Racks of cone-tainers were rotated (within their assigned light treatment) in the greenhouse every two weeks for the duration of the study.

Experimental Design

The experimental design is shown graphically in **Figure 1**. Each field-collected maternal family was represented by three siblings. The three siblings were separated into three groups (A, B, C). One sibling per family was designated as a 'pollen recipient' (individuals in Group C) and the other two as 'pollen donors' (individuals in Groups A and B). Pollen recipients (Group C) were grown under Lumigrow Pro LED lights (lumigrow.com) for 10 h a day for the duration of the study. As soon as the first flower opened on each individual pollen recipient, we removed both sets of anthers to prevent self-fertilization. Pollen recipients were physically separated from pollen donors in the greenhouse to prevent accidental cross-pollination (**Figure 1**).

The two pollen donor siblings were separated into two groups (A and B). Plants in group A were grown exclusively under Lumigrow Pro LED lights for 10 h a day. Plants in group B were grown under standard Lumigrow Pro LED lights and an additional UV lamp (Exo Terro Sunray^{TM},[3]) for 10 h a day. This additional lamp included light in the UV spectrum (300–400 nm) in addition to light in the visible spectrum. Plants in experimental light group B were exposed to both UV light and visible range light. We did not account for differences in the amount of visible light (green, blue, red light) between experimental groups A and B. In some species, UV radiation has been shown to be an important regulating factor of anthocyanin production and petal color formation (Llorens et al., 2015). The greenhouse glass filtered out the majority of UV light; however, it is likely that UV light was not 100% excluded from the individuals in Group A. We included light treatment (A or B) in our analyses

[1] www.stueweandsons.com

[2] www.islandseed.com

[3] www.exo-terra.com

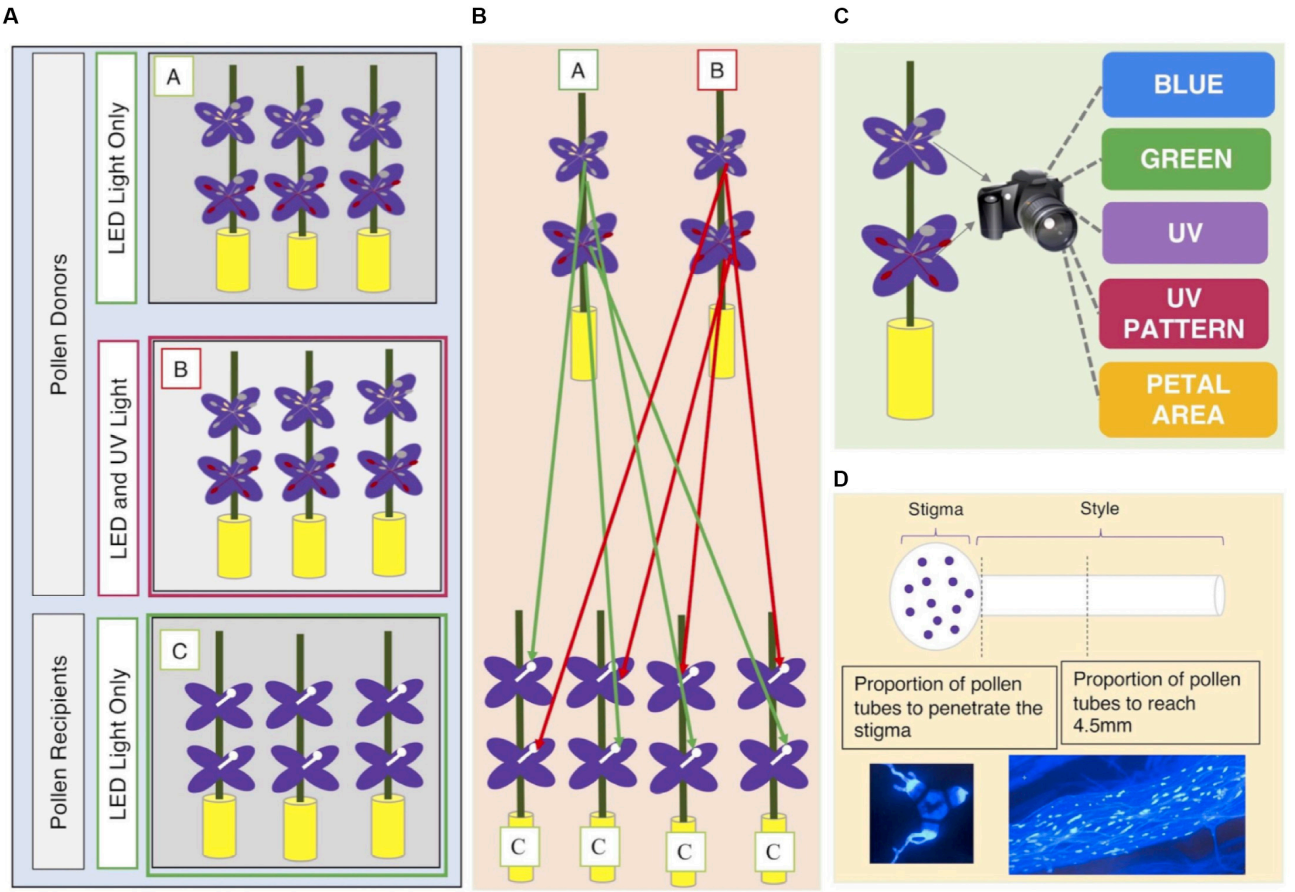


FIGURE 1 | (A) Seeds from 35 maternal families per population were collected from eight wild populations of C. unguiculata. Three seedlings per maternal family were cultivated and assigned a group (A, B, C). Individuals in Group C were designated as pollen recipients. Pollen recipients (Group C) were grown under Lumigrow Pro LED lights for 10 h/day. Individuals in Groups A and B were designated as pollen donors. Pollen donors in Group A were also grown under Lumigrow Pro LED lights for 10 h/day. Pollen donors in Group B were grown under Lumigrow Pro LED lights and an additional full spectrum lamp (Exo Terro Sunray www.exo-terra.com) for 10 h a day. **(B)** We harvested pollen from both anther types from each pollen donor plant. From each pollen donor flower, we harvested pollen from the short set of anthers and, from another flower on the same plant, the long set of anthers to perform 1-3 hand pollinations (per set of anthers) on separate pollen recipient flowers (on separate pollen recipient plants). All hand pollinations were conducted using donors and recipients from the same population. **(C)** At the same time that we removed the anthers from each pollen donor flower we removed one petal. We took a multispectral image of the flower petal (see section "Materials and Methods"). **(D)** Four hours after each hand-pollination, we collected the stigma and style by severing the style at its base with a scalpel. We placed each stigma and style in a microcentrifuge tube filled with formalin acetic acid to arrest pollen tube growth. Staining with aniline blue and the use of a fluorescence microscope (with a DAPI filter) allowed us to visualize and count the pollen grains adhering to the stigma and the progress of pollen tubes down the style.

for two reasons: first, to determine whether plants that were exposed to supplemental UV light produced more anthocyanins than their siblings that were exposed only to LED lighting; and, second, to determine the independent effects of supplemental UV light, anthocyanin concentration, and UV nectar guide size on pollen performance.

Climate Data

To determine the effects of specific features of climate on flower color, pattern, and size, we extracted historic climate normals for each population using ClimateNA (Wang et al., 2016). This software extracts and downscales 1961-1990 monthly climate normal data from a spatial resolution of 4 × 4 km and calculates many monthly, seasonal and annual climate variables for specific locations based on latitude, longitude, and elevation (Wang et al., 2016). We extracted the mean values (from 1961 to 1990) of the following climatic variables for each population: mean temperature of the warmest month (C°), mean annual precipitation (mm), and summer heat moisture index. The summer heat:moisture index is a scaled, averaged (over a decade) ratio between mean warmest month temperature and mean summer precipitation. Hot, dry years/locations have relatively high indices, while cool, wet locations have relatively low indices.

For each site, we also extracted the sum of the solar radiation of the highest quarter (J/m^2/day) for each population (Sum UV) from glUV, a global UV-B radiation dataset for macroecological studies (Beckmann et al., 2014), to use as a proxy for levels of solar UV radiation during the flowering period of *C. unguiculata*. glUV was generated using UV-B data from

the Ozone Monitoring Instrument (OMI) onboard the NASA EOS Aura spacecraft (Beckmann et al., 2014). OMI contains two spectrometers and measures reflected solar radiation in a selected range of the visible (350–500 nm) and UV light spectrum (Levelt et al., 2006). The OMI measurements are used to calculate clear-sky surface UV irradiance, which is corrected for clouds and aerosols to obtain the OMI surface UV irradiance values (Tanskanen et al., 2006). The accuracy of these measurements, assessed with ground-reference data, ranges from 70% to 93%, depending on atmospheric and location-specific conditions (Tanskanen et al., 2006).

Pollination Study

We harvested pollen from both anther types (see section "Study Species") produced by each pollen donor in Groups A and B (n = 399). From each pollen donor flower, we harvested pollen from the short set of anthers and, from another flower on the same plant, the long set of anthers. These pollen samples were used to hand-pollinate 1-3 flowers (per set of anthers) on 1-3 separate pollen recipient flowers (see **Figure 1**). Each recipient flower received pollen from only one donor and one anther type. The inner (short) series of anthers produced only enough pollen to cover the surface of one stigma, so each set of short anthers was typically used in only one hand pollination. In all cases, the pollen recipients were from a different maternal family than the pollen donor plant. All hand pollinations were conducted using donors and recipients from the same population.

To perform each pollination, we selected the pollen donor flower and recorded the donor's Individual ID number, floral sequence, anther type used, and light treatment. From this flower, we then removed one entire petal. We took a multispectral image of the petal using the method described below (see also **Supplement 3**). From the flower, we then removed the anthers of the type specified in the previous step and placed them in a microcentrifuge tube. Pollen grains were dislodged from the anther surface by agitating the closed microcentrifuge tube. We used a dissecting spatula to cover the stigmatic surface of an emasculated flower of the assigned pollen recipient with pollen from a single set of anthers. Four hours after each hand-pollination, we removed the stigma and style by severing the style at its base with a scalpel and placed each stigma and style in a microcentrifuge tube filled with formalin acetic acid to arrest pollen tube growth. Microcentrifuge tubes were labeled with an identification number linked to the pollen donor, pollen recipient, and light treatment of the associated sample. We also recorded the floral sequence of each pollinated flower on the primary stem of each pollen recipient. The floral sequence refers to the number of flowers produced before the focal flower, inclusive of the first flower (e.g., the first flower produced by a plant has a floral sequence of one). From among the total of ∼700 hand-pollinated flowers, pollen performance was unambiguously scored for 398 flowers (n = 230 pollinations using long-anther pollen and n = 168 pollinations using short-anther pollen).

Previous studies have shown that pollen germination rates and pollen tube growth rates are sensitive to temperature (Hedhly et al., 2009; Hove and Mazer, 2013; Mazer et al., 2018). Accordingly, we recorded the mean temperature of the greenhouse hourly during the four-hour window following each hand-pollination. For each flower, the mean greenhouse temperature recorded during the four-hour pollination window is included as an independent variable in the statistical analyses described below.

Pollen Performance

We used the methods described by Martin (1959) to soften and clear the styles and to stain them with aniline blue. This process allows the visualization and counting of pollen grains and pollen tubes using a fluorescence microscope. A negative effect of pollen load on the proportion of pollen grains that penetrate the stigma due to gametophyte competition for either space or maternal resources has been demonstrated in *C. unguiculata* (Mazer et al., 2016). Therefore, to control for the potential effects of variation in the intensity of early-stage pollen competition on pollen performance, we counted the number of pollen grains adhering to each stigmatic surface and included this value as an independent variable in the models described below. Given that some of the pollen deposited on the stigma may have been dislodged when placed in solution in the microcentrifuge tube after they were harvested, the number of pollen grains adhering to the surface provides a measure of the number of grains that became anchored to the stigma within four hours of pollination (likely due to features of the pollen wall or germination) and are competing for access to the stigma. We quantified pollen performance by determining the proportion of these pollen grains that penetrated the stigma surface (PSP, proportion stigma penetrance) and the proportion whose tubes successfully grew to a distance of 4.5mm down the style (P4.5, the proportion of tubes to reach 4.5mm from the stigma surface) within the 4-hour post-pollination period. We selected 4.5mm because it represents ∼25% of the mean style length of this species (Peach and Mazer, 2019). We were able to quantify these metrics by examining the aniline blue stained stigma and style under a fluorescence microscope (with a DAPI filter) and counting the number of callose plugs deposited in the specified region of the pistil (cf. Hove and Mazer, 2013; Mazer et al., 2016; Mazer et al., 2018).

Multispectral Photography and Image Analysis

When used properly, digital cameras are useful and relatively inexpensive tools with which to quantify color and pattern (Stevens et al., 2007; Spottiswoode and Stevens, 2010; Macfarlane and Ogden, 2012; Garcia et al., 2014; Troscianko and Stevens, 2015). Digital photography has been used in several studies to measure and to characterize plant and animal coloration, pattern, and camouflage (Strauss and Cacho, 2013; Stevens et al., 2017). In a related application of this approach, Del Valle et al. (2018) developed an efficient, non-invasive method for estimating anthocyanin concentration using red, green, and blue reflectance values extracted from digital images.

We used multispectral photography and image analysis to extract objective measurements of color-specific reflectance

values and UV pattern information, petal area, and anthocyanin concentration of the flowers of *C. unguiculata.* Because digital cameras were initially designed to generate photographs for human vision, the sensors in "off the shelf" cameras typically come equipped to prevent ultraviolet (UV) and infrared light from hitting the sensor. This buffering can be removed, creating a "full spectrum" camera with a sensor that receives incoming light at all wavelengths. To quantify complex variation in color-specific reflectance, we converted a Panasonic LUMIX GX7 digital camera with its LUMIX 14-42mm II lens[4] (Kadoma, Osaka Prefecture, Japan) to a 'full spectrum' camera using LifePixel conversion services[5] (Mukilteo, WA, United States) and then analyzed the resulting images using an ImageJ software plug-in (originally created to characterize cuckoo eggshell patterns by Troscianko and Stevens, 2015). The camera lens used in this study transmits UV light that is received by the camera sensor.

There are other camera lenses (e.g., UV CoastalOpt® SLR objective, or Nikon 105/4.5 UV Nikkor objective lens[6]) that are specifically designed to transmit UV light and that are capable of generating sharper UV images than the Lumix lens used here.

Given that our lens may not have transmitted 100% of UV light available, our measurements of UV mean petal reflectance may be underestimates. Recording absolutely accurate values of this trait (UV mean reflectance), however, was not required to address our primary questions, which required only that we obtain accurate relative values of UV mean petal reflectance within and between populations, which our camera provided. Consequently, the red, blue, and green reflectance measurements and UV nectar guide size reported here may all be interpreted as objective measures of petal color reflectance and size.

The Lumix GX7 comes with a Four Thirds (17.3 × 13 mm) CMOS sensor, which has a diagonal of 21.64 mm (0.85″) and a surface area of 224.90 mm^2. Exposure and metering are easily manipulated in this camera model, which is required for unbiased data acquisition (Stevens et al., 2007). The Lumix GX7 is a mirrorless digital camera, which can calculate the optimal exposure under fixed lighting conditions using the "A" mode (aperture priority) setting (even in UV conditions) (Troscianko and Stevens, 2015). Once the optimal aperture was selected, the aperture setting remained fixed for the duration of the study and the camera automatically selected the best shutter speed. To ensure the capture of a perfectly exposed image, we used "exposure bracketing" whereby the camera automatically adjusts the exposure range across three photos to intentionally under- and over-expose the image by a set number of "exposure values". Images were reviewed for acceptable exposure using the histograms available in the RawTherapee application.[7] To calibrate each image to estimate petal area a ruler was included at a fixed position in every photograph. All images were captured in RAW format, which is required for reliable image extraction. RAW files contain unprocessed images which may be linearized using specialized software (The Multispectral Image Analysis Toolbox).

Both photos were taken at a station that included the full spectrum modified camera mounted on a tripod with a locked camera position and a standardized light source (a single 70 W Exo terro sunray, www.exo-terra.com, Mansfield, MA, United States). The height and the angle of the light source were not altered between photographs. We used two lens filters alternately to produce two images (which were later merged in ImageJ into a single multispectral image). The first image was created using a Baader U 2″ UV-pass filter (www.baader-planetarium.com, Mammendorf, Germany), which can be manually added to the camera to allow only ultraviolet (UV) light (300–400 nm with peak permeability at 350 nm) to hit the sensor, thereby generating a UV image. To create the second image, we replaced the UV-pass filter with a UV/Infrared light blocking filter[8] (UVR Defense Tech, United States), which allows only visible green, blue and red light to pass through and hit the camera sensor.

We calibrated digital photographs to allow their use for objective measurements of color or pattern within or between photographs (Stevens et al., 2007). We used the "Multispectral Image Analysis Toolbox" (Troscianko and Stevens, 2015), which is a plugin for ImageJ (Schneider et al., 2012). Several studies have used this toolkit (Stevens et al., 2017; Gómez et al., 2018; van den Berg et al., 2019; Hawkes et al., 2019; Price et al., 2019; Šulc et al., 2019), which has many advantages including; easy linearization, high precision, and low data loss in image analysis due to 32-bit floating-point image processing (Troscianko and Stevens, 2015). The image frame included a fixed position ruler and Spectralon™ diffuse reflectance standard with a flat reflectance value of 20% across the entire light spectrum, including red, blue, green, and UV wavelengths[9] (North Sutton, NH, United States). For image calibration, we selected the Spectralon™ diffuse reflectance standard (20% reflectance, Troscianko and Stevens, 2015) and used the setting for "visible + UV" photography and "aligned normalized 32-bit" files. This calibration process successfully linearized the RGB and UV values in each photograph.

The Multispectral Image Analysis Toolbox plugin (Troscianko and Stevens, 2015) for ImageJ (Schneider et al., 2012) combines red, green, blue, and UV wavelengths into one multispectral image and facilitates the extraction of objective measurements of color-specific reflectance values and UV pattern information. UV nectar guide area was determined by viewing the multispectral image in the UV channel and using existing ROI (Region of Interest) tools in ImageJ to draw a line along the border of the visually apparent UV-absorbing nectar guide in the claw of the petal. Objective measurements of mean UV (300–400 nm), blue (430–500 nm), green (510–530 nm) and red (560–580) reflectance were recorded for each petal analyzed (n = 700). We measured these wavelengths specifically due to their role in pollinator attraction (Peach et al., 2020) and to estimate anthocyanin

[4]www.panasonic.com

[5]www.lifepixel.com

[6]https://www.jenoptik.us/

[7]https://rawtherapee.com/

[8]www.uvroptics.com

[9]www.labsphere.com

concentration (see below). The area (mm^2) of each region was also recorded. Image acquisition methods are described in detail in **Supplement 3**.

Estimation of Anthocyanin Concentration

Anthocyanins are present in most plant organs and are usually stored in vacuoles of epidermal cells (Lee and Finn, 2007; Gould et al., 2008; Landi et al., 2015). Anthocyanins absorb light at specific wavelengths, and the remaining light is reflected outward, which produces the visible colors in wavelengths of 400 – 700 nm (Kay et al., 1981; van der Kooi et al., 2016). When color-specific reflectance values (red, blue, green) are calculated for plant tissues using digital photography and multispectral image analysis (described above), there are several equations that can be used to estimate anthocyanin concentration without destructive sampling (Del Valle et al., 2018). Del Valle et al. (2018) found that color-specific reflectance values extracted from digital images in the visible light range can be used to accurately estimate anthocyanin concentration in floral tissues. We took multispectral images of flower petals to quantify their anthocyanin concentration using this method. To estimate anthocyanin concentration, we extracted only red, blue, and green reflectance values from "visible range" digital images. We did not use UV reflectance values to calculate anthocyanin concentration. We calculated anthocyanin concentration using the "S_green" equation described by Del Valle et al. (2018), which was reported by the authors to provide estimates of anthocyanin concentrations that are slightly more accurate than spectrophotometry. Total amounts of anthocyanins are reported as absorbance units (AU) per cm^2 of fresh material.

Statistical Analysis

Geographic variation in petal color, pattern, and size

We calculated the population mean for each light treatment and functional sex stage. We treated each functional sex stage as distinct because in previous work we found that functional sex is a significant source of variation in these floral traits (Peach et al., 2020). Using these population means (8 populations × 2 light treatments × 3 functional sex stages = 48 mean values) of each of our focal floral traits as a dependent variable, we conducted several stepwise OLS (ordinary least squares) regressions to detect the effects of floral functional sex stage (male stage 1, male stage 2, and female [stage 3]), light treatment (LED vs. LED + UV), latitude, elevation, mean temperature of the warmest month (C°) (MTWM), mean annual precipitation (mm) (MAP), summer heat moisture index (SHMI), the sum of the solar radiation of the highest quarter (J/m^2/day) (SumUV) and all two-way interactions. We calculated variance inflation factors for all of our bioclimatic predictor variables and identified problematic multicollinearity. Our goal was to identify the independent effects of each bioclimatic predictor on our focal floral traits, which we would be unable to do if collinear variables were included in the same model. We split the analyses of our bioclimatic predictors into two models, such that within each model there was no high collinearity between any of the predictor variables (all variance inflation factors of main effects were < 5). By splitting the analysis into two models, each model's output avoided unreliable parameter estimation due to multicollinearity among its included variables. Variance inflation factors are reported with the parameter estimates in **Supplement 4**.

The predictor variables included in the final model were also used to construct an ANOVA (conducted using Type III sums of squares) to determine the independent effects of each predictor variable/interaction on each focal floral trait. We used Type III sums of squares (SS) to test for the effects of our bioclimatic predictors on each focal floral trait when each variable was placed last into the model; this tests for the effect of each variable independent of all others included in the model. Adjusted partial R^2 values were calculated using the 'rsq.partial' function in the rsqv1.1 package in R studio.

Testing the protective function of UV floral patterns

We scored pollen performance within the 398 hand-pollinated flowers. Each data point in this analysis represents the pollen performance measurements from a single hand-pollinated flower (not a population mean). For each measure of pollen performance (PSP and P4.5), we conducted a stepwise OLS regression to detect the effects of anther type (long vs. short), light treatment, floral sequence, greenhouse temperature (during pollen germination and growth), anthocyanin concentration, proportion nectar guide, UV mean petal reflectance, and all two-way interactions. We conducted two additional stepwise OLS regressions (one for each pollen performance trait) to detect the effects of anther type, light treatment, floral sequence, greenhouse temperature, MTWM, MAP, SHMI, SumUV and all two-way interactions. We also constructed an ANOVA (conducted using Type III SS) to determine the independent effects of each predictor variable and interaction on pollen performance. All analyses were conducted in R Studio version 1.2.1335 (Lüdecke, 2019).

RESULTS

Petal Color and Size

Experimental light treatment and several environmental variables had direct effects on petal color. Supplemental UV light (Light treatment B) had a significant negative effect on anthocyanin concentration but did not affect any other trait measured in this study (**Table 1**). Latitude and SumUV of the sampled populations' locations had significant positive effects on anthocyanin concentration and mean UV petal reflectance (**Table 1** and **Figures 2**, **3**). The interaction between latitude and SumUV also had a significant effect on anthocyanin concentration and mean UV petal reflectance (**Supplement 5**). The slope of the relationship between SumUV and anthocyanin concentration was significant only at extreme latitudes (very low or very high). MAP and elevation had significant negative effects on anthocyanin concentration (**Table 1** and **Figure 3**). Petal area (mm^2) decreases with increasing latitude and SumUV (**Table 1** and **Figure 3**).

Model parameter estimates, and standard errors are reported in **Supplement 4**.

UV Floral Pattern

To our knowledge, this is the first study to describe the presence of UV-absorbing floral patterns (nectar guides) in *C. unguiculata*. Mean proportion nectar guide ranged from 0.05–0.57 (± SE 0.002, CV = 41.31) across populations and light treatments. Across *C. unguiculata*'s sampled range, latitude and SumUV had significant negative effects on the proportion nectar guide (latitude $\beta = -0.343 \pm SE = 0.125$, SumUV $\beta = -0.0008 \pm SE = 0.0003$) and nectar guide area (latitude $\beta = -11.759 \pm SE = 2.986$, SumUV $\beta = -0.026 \pm SE = 0.007$) (**Table 1**, **Figure 1** and **Supplement 4**). This pattern was consistent using multiple measurements of UV including SumUV, mean summer solar radiation, and mean annual solar radiation. All three of these measurements of solar UV were strongly positively correlated, so only one (SumUV) was selected for the analyses presented here.

Among the populations examined here there was no correlation between anthocyanin concentration and proportion nectar guide ($P = 0.87$) or anthocyanin concentration and mean UV petal reflectance ($P = 0.69$). If the geographic patterns observed in this study were artifacts of a shared developmental pathway between UV-absorbing flavonoids and anthocyanin-flavonoids we would expect a strong negative correlation between these variables.

TABLE 1 | (a–c) Summary of multivariate models to detect the independent effects of light treatment, floral sex stage, latitude, temperature, and solar UV on proportion nectar guide, anthocyanin concentration, mean UV petal reflectance, nectar guide area and petal area in *C. unguiculata*.

(a)	**Proportion nectar guide**				**Anthocayanin concentration**			
	SS	**DF**	***F*-value**	**Pr(>F)**	**SS**	**DF**	***F*-value**	**Pr(>F)**
(Intercept)	0.009	1	7.771	0.008	3.544	1	8.982	0.005
Light treatment	1.00E-04	1	0.096	0.758	3.428	1	8.689	0.005
Floral stage	0.004	2	1.893	0.164	5.541	2	7.022	0.002
Latitude	0.008	1	7.516	0.009	3.573	1	9.056	0.005
Mean temperature of the warmest month (°C)	0.007	1	6.776	0.013	0.202	1	0.512	0.478
Sum of the solar radiation of the highest quarter (J/m^2/day)	0.008	1	7.746	0.008	3.667	1	9.296	0.004
Latitude:Sum of the solar radiation of the highest quarter (J/m^2/day)	0.008	1	7.656	0.009	3.858	1	9.779	0.003
Model	0.04	40	2.367	0.04	15.781	40	11.3	< 0.0001
Adjusted R^2				0.17				0.61
(b)	**Mean UV petal reflectance**				**Nectar guide area**			
	SS	**DF**	***F*-value**	**Pr(>F)**	**SS**	**DF**	***F*-value**	**Pr(>F)**
(Intercept)	3.849	1	11.153	0.002	9.099	1	15.76	0.0003
Light treatment	0.983	1	2.849	0.09	1.00E-05	1	0.00	0.997
Floral stage	0.625	2	0.905	0.413	8.888	2	7.697	0.001
Latitude	3.482	1	10.086	0.003	8.953	1	15.508	0.0003
Mean temperature of the warmest month (°C)	10.381	1	30.072	2.51	3.357	1	5.814	0.02
Sum of the solar radiation of the highest quarter (J/m^2/day)	3.699	1	10.718	0.002	9.099	1	15.761	0.0003
Latitude:Sum of the solar radiation of the highest quarter (J/m^2/day)	3.208	1	9.294	0.004	9.025	1	15.632	0.0003
Model	13.808	40	13.74	< 0.0001	23.094	40	5.915	0.0001
Adjusted R^2				0.65				0.42
(c)	**Petal area**							
	SS	**DF**	***F*-value**	**Pr(>F)**				
(Intercept)	0.996	1	5.421	0.025				
Light treatment	0.02	1	0.11	0.742				
Floral stage	28.711	2	78.104	1.54E-14				
Latitude	0.943	1	5.132	0.029				
Mean temperature of the warmest month (°C)	0.225	1	1.224	0.275				
Sum of the solar radiation of the highest quarter (J/m^2/day)	0.919	1	5.002	0.031				
Latitude:Sum of the solar radiation of the highest quarter (J/m^2/day)	0.915	1	4.978	0.031				
Model	7.352	40	30.82	< 0.0001				
Adjusted R^2				0.82				

(Continued)

TABLE 1 | (d–f) Summary of multivariate models to detect the independent effects of light treatment, floral sex stage, precipitation, and elevation on proportion nectar guide, anthocyanin concentration, mean UV petal reflectance, nectar guide area and petal area in *C. unguiculata*. SS = Sum of squares.

(d)	Proportion nectar guide				Anthocyanin concentration			
	SS	DF	*F*-value	Pr(>F)	SS	DF	*F*-value	Pr(>F)
(Intercept)	0.0001	1	0.142	0.708	10.006	1	15.315	0.0003
Light treatment	1.00E-04	1	0.103	0.75	3.428	1	5.247	0.03
Floral stage	0.004	2	2.026	0.145	5.541	2	4.24	0.02
Mean annual precipitation (mm)	0.012	1	12.974	0.001	11.022	1	16.87	0.0002
Elevation (m)	0.009	1	9.161	0.004	8.811	1	13.486	0.0007
Mean annual precipitation (mm):Elevation (m)	0.01	1	10.657	0.002	10.193	1	15.601	0.0003
Model	0.039	41	3.258	0.01	26.789	41	5.156	0.0005
Adjusted R^2				0.22				0.35
(e)	**Mean UV petal reflectance**				**Nectar guide area**			
	SS	DF	*F*-value	Pr(>F)	SS	DF	*F*-value	Pr(>F)
(Intercept)	0.132	1	0.26	0.613	10.474	1	16.103	0.0002
Light treatment	0.983	1	1.929	0.172	1.00E-05	1	0.00	0.997
Floral stage	0.625	2	0.613	0.547	8.888	2	6.832	0.003
Mean annual precipitation (mm)	0.467	1	0.917	0.344	11.303	1	17.377	0.0002
Elevation (m)	0.829	1	1.627	0.209	9.312	1	14.316	0.0005
Mean annual precipitation (mm):Elevation (m)	0.083	1	0.163	0.689	10.508	1	16.155	0.0002
Model	20.902	41	8.532	< 0.0001	5.21	41	26.668	0.0004
Adjusted R^2				0.49				0.35
(f)	**Petal area**							
	SS	DF	*F*-value	Pr(>F)				
(Intercept)	0.325	1	0.81	0.373				
Light treatment	0.02	1	0.05	0.823				
Floral stage	28.711	2	35.779	1.02				
Mean annual precipitation (mm)	0.403	1	1.004	0.322				
Elevation (m)	0.59	1	1.47	0.232				
Mean annual precipitation (mm):Elevation (m)	0.678	1	1.689	0.201				
Model	16.45	41	12.69	< 0.0001				
Adjusted R^2				0.6				

The interaction between SumUV and latitude had a significant effect on proportion nectar guide and nectar guide area. The slope of the relationship between SumUV and proportion nectar guide was significant only at relatively extreme latitudes. See **Supplement 4** for a visualization of the effects of this interaction on UV floral patterns. MTWM, MAP and elevation had significant positive effects on proportion nectar guide and nectar guide area (**Table 1**, **Supplement 4**).

Pollen Performance

Anther type, floral position, pollen load, greenhouse temperature, and environmental conditions of the sampled field sites all influenced pollen performance. Anthocyanin concentration and proportion nectar guide, however, did not influence pollen performance under either light treatment, independent of variation in the other variables included in the model (**Table 2** and **Figure 4**). The anther type from which the pollen was collected had a significant effect on both PSP and P4.5. Pollen harvested from the shorter series of anthers performed better than pollen harvested from the longer series (PSP $\beta = 0.019 \pm SE = 0.008$, P4.5 $\beta = 0.044 \pm SE = 0.012$). The position of the pollen donor's flower from which pollen was used had a significant positive effect on the PSP; pollen produced by relatively distal flowers (those with higher floral sequence values) had higher PSP than pollen produced by relatively basal flowers ($\beta = 0.006 \pm SE = 0.009$). Pollen load had a significant negative effect on the PSP ($\beta = -0.0002 \pm SE = 0.00004$, **Table 2**, **Supplement 4**). Mean greenhouse temperature did not have a significant effect on PSP or P4.5. Pollen performance also differed among populations sampled from environmentally distinct geographic regions. Among the field sites from which seeds were collected for this experiment, SumUV had a significant positive effect on PSP ($\beta = 0.00003 \pm SE = 0.00001$) but no effect on P4.5. Parameter estimates for interaction terms are reported in **Supplement 4**.

DISCUSSION

The tremendous diversity of floral form has held the interest and imagination of biologists for centuries. Evolutionary biologists frequently consider the diversification of flowering plants to

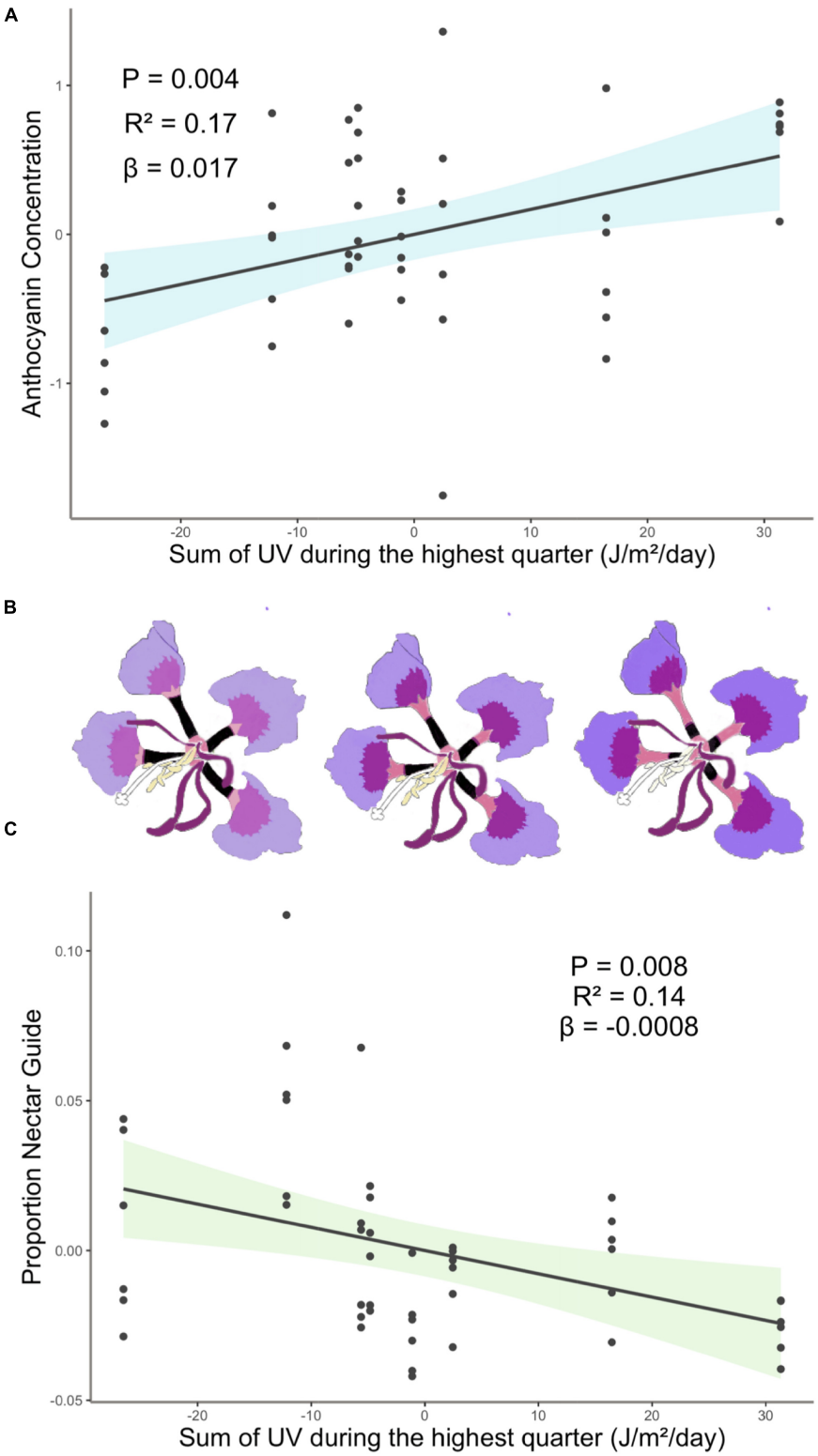


FIGURE 2 | (A) Anthocyanin concentration was highest in populations with high SumUV **(B)** Representative depiction of geographic variation in estimated mean floral anthocyanin concentration and proportion nectar guide of eight populations of *C. unguiculata.* UV-absorbing nectar guides begin at the base of the claw and extend up each claw toward the blade. In this figure they are depicted as black rectangles, which is similar to how they appear in UV photos. **(C)** Proportion nectar guide was largest in low SumUV populations. Shaded area indicates the 95% confidence interval. β = the regression coefficient associated with the independent variable in each panel. *P* = the *P*-value associated with the regression coefficient. R^2 = the partial r squared associated with the independent variable in each panel.

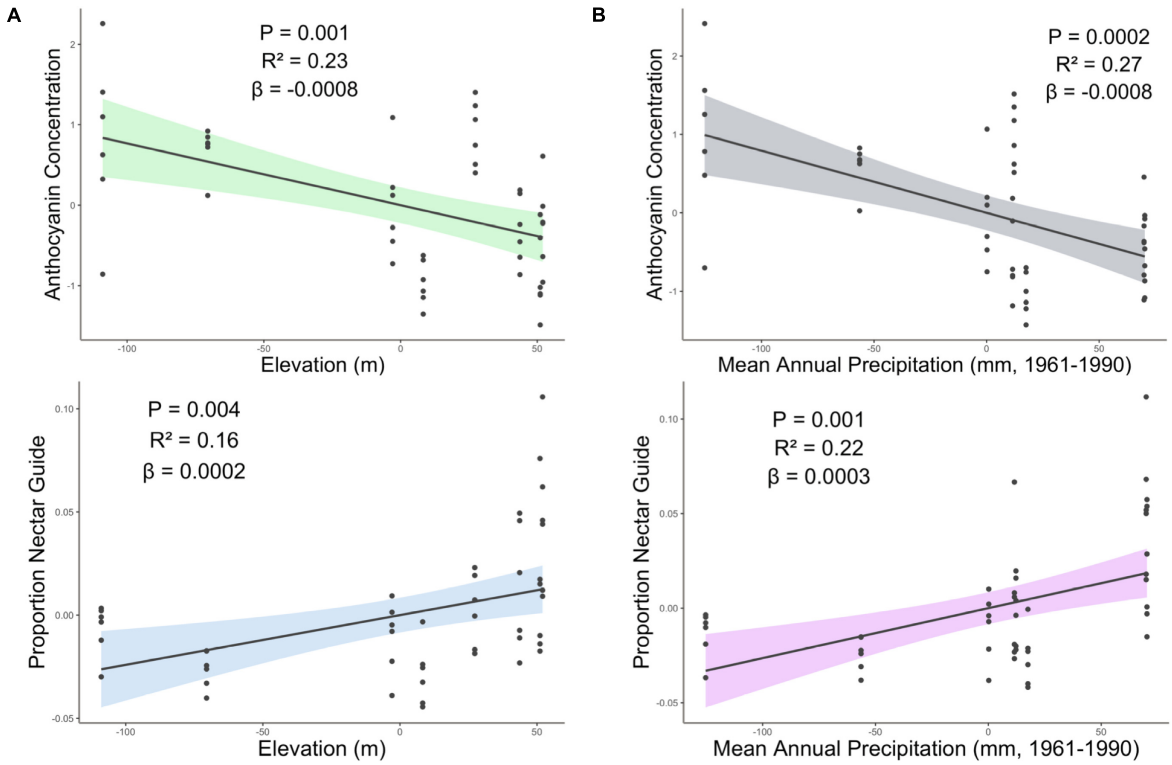


FIGURE 3 | Partial regression plots derived from linear models showing the effect of **(A)** elevation and **(B)** precipitation on proportion nectar guide and anthocyanin concentration. Shaded area indicates the 95% confidence interval. β = the regression coefficient associated with the independent variable in each panel. *P* = the *P*-value associated with the regression coefficient. R^2 = the partial r squared associated with the independent variable in each panel.

be primarily driven by a combination of variation in abiotic (habitat and landform) and biotic (pollinator choice, availability and efficiency) conditions (Harder and Barrett, 2006; Rausher, 2008). Geographic variation in floral traits across heterogeneous landscapes provides the opportunity to examine the process and outcome of natural selection in the wild (Whittall and Strauss, 2006; Tripp et al., 2018).

The climate of North America will change dramatically over the coming decades, altering the selective pressures that influence the evolution of floral traits in wild populations (IPCC AR4, 2007; Christiansen et al., 2011). Understanding the mechanisms by which plants have responded to existing variation in climate can broaden our knowledge of evolutionary processes and inform estimates of how populations and species may respond to climate change (Harder and Barrett, 2006; Hoffmann and Sgrò, 2011.). The large geographic range of *Clarkia unguiculata* provides a rich mosaic in which to study the factors associated with the evolutionary diversification of floral traits beyond the traditional plant-pollinator framework.

Is Intraspecific Variation in Floral Color and Pattern Geographically Structured in This Species?

In this study we found that intraspecific variation in floral color, pattern, and size is geographically structured and that several components of climate (and topography) explain a significant proportion of variation in these traits. However, only some of the geographic patterns reported here are consistent with those observed in other species (Koski and Ashman, 2015; del Valle et al., 2015; Berardi et al., 2016).

Latitude and elevation are often used as a proxy for a suite of abiotic conditions (Nybakken et al., 2004; Körner, 2007; Berardi et al., 2016; Bergamo et al., 2018). In this study we used long-term climate normals for several parameters to determine which climate features are correlated with flower pigment, pattern, and size in *C. unguiculata*. We included latitude and elevation in the models because they may serve as a proxy for one or more unmeasured factors. Pollinator assemblage or abundance, humidity, or herbivore/florivore pressure are all examples of factors that may be associated with latitude or elevation but were not accounted for directly here (Arnold et al., 2009; Jaakola and Hohtola, 2010; Tsuchimatsu et al., 2014; Berardi et al., 2016). For example, in some plant species, high anthocyanin concentration is associated with reduced herbivory (Irwin et al., 2003; Gould, 2004; Hanley et al., 2009). If herbivore pressure is more intense at lower elevations/latitudes and anthocyanin concentration deters herbivory in *C. unguiculata* (as it does in other species), then this may explain the negative correlation between elevation/latitude and anthocyanin concentration observed here. However, we do not have detailed

TABLE 2 | (a) Summary of multivariate models to detect the independent effects of anther type, greenhouse temperature, pollen load, floral sequence, SumUV and MTWM on pollen performance in *C. unguiculata*. **(b)** Summary of multivariate models to detect the independent effects of anther type, greenhouse temperature, pollen load, floral sequence, proportion nectar guide, anthocyanin concentration and mean UV petal reflectance on pollen performance in *C. unguiculata*. SS = Sum of squares.

(a)

	Proportion of pollen tubes to penetrate the stigma (PSP)				Proportion of pollen tubes to reach 4.5 mm from the base of the stigma (P4.5)			
Term	**DF**	**SS**	**F Ratio**	**Pr(>F)**	**DF**	**SS**	**F Ratio**	**Pr(>F)**
Anther Type	1	0.12	6.567	0.011*	1	0.544	11.961	0.001*
Mean Greenhouse Temperature (°C)	1	0.383	20.888	<0.0001*	1	0.004	0.098	0.755
Anther Type * Mean Greenhouse Temperature	1	0.489	26.71	<0.0001*	1	0.009	0.203	0.652
Pollen Load	1	0.647	35.296	<0.0001*	1	0.297	6.539	0.011*
Floral Sequence of the Pollen Donor	1	0.952	51.932	<0.0001*	1	0.056	1.232	0.268
Sum of the UV of the highest quarter (J/m^2/day)	1	0.185	10.106	0.002*	1	0.083	1.823	0.178
Mean temperature of the warmest month (°C)	1	0.076	4.151	0.042*	1	0.199	4.369	0.037*
Anther Type* SumUV	1	0.113	6.139	0.014*	1	0.009	0.197	0.657
Model	8	3.315	22.61	<0.0001*	8	1.866	5.131	<0.0001*
Error	389	7.13			389	17.685		
C. Total	397	10.445			397	19.551		
Adjusted R^2				0.3				0.08

(b)

Anther Type	1	0.139	7.318	0.007*	1	0.618	13.178	0.0003*
Mean Greenhouse Temperature (°C)	1	0.226	11.895	0.001*	1	0.065	1.382	0.241
Anther Type * Mean Greenhouse Temperature	1	0.372	19.588	<0.0001*	1	0.014	0.291	0.59
Pollen Load	1	0.577	30.376	<0.0001*	1	0.336	7.166	0.008*
Floral Sequence of the Pollen Donor	1	0.936	49.256	<0.0001*	1	0.003	0.068	0.794
Proportion Nectar Guide	1	0.041	2.173	0.141	1	0.011	0.242	0.623
Anthocyanin concentration	1	0.002	0.128	0.721	1	0.043	0.906	0.342
Mean UV Petal Reflectance	1	0.001	0.072	0.789	1	0.0199	0.425	0.515
Model	8	3.08	20.262	<0.0001*	8	1.319	3.513	0.001*
Error	386	7.333			386	18.116		
C. Total	394	10.413			394	19.435		
Adjusted R^2				0.28				0.05

records of herbivore assemblage or abundance for all of the locations examined in this study.

Which Floral Traits Are Best Predicted by Components of Climate and Geography in *C. unguiculata*?

Anthocyanin Concentration

Flavonoids are ubiquitous plant secondary metabolites. The best-known examples are the characteristic red, blue, and purple anthocyanin pigments of plant tissues (Winkel-Shirley, 2001; Grotewold, 2006). Flavonoids are important secondary metabolites that help to protect plants against UV radiation and other forms of abiotic stress (Burns, 2015; Llorens et al., 2015). Given the well-documented increase in solar radiation (UV) toward the equator, plant flavonoid production is expected to increase with decreasing latitude (Jaakola and Hohtola, 2010; Llorens et al., 2015; Tripp et al., 2018). However, some studies have shown that anthocyanin flavonoids may exhibit the opposite geographic pattern (Hårdh and Hårdh, 1977; Lätti et al., 2008). Studies of *Vaccinium myrtillus* (northern bilberry) (Lätti et al., 2008) and *Fragaria virginiana* (wild strawberry) (Hårdh and Hårdh, 1977) report that populations at higher latitudes were characterized by higher concentrations of anthocyanin than those at lower latitudes.

Our results did not corroborate this expected pattern. Many of the factors that purportedly influence flower color and size are often tightly correlated with each other (e.g., locations at low latitudes often have high solar UV) making it difficult to identify the proximate agents of selection. The coast of California is characterized by a high density of coastal fog for several months of the year, resulting in a latitudinal gradient that includes unique combinations of climate, such as high-UV and high latitude (Torregrosa et al., 2016). Among the populations of *C. unguiculata* sampled here, those growing closer to the equator were not characterized by petals with a higher concentration of anthocyanin than those at higher latitudes. However, populations characterized by high UV conditions were associated with higher mean anthocyanin concentration than those in low UV conditions (**Figure 2**). The pattern

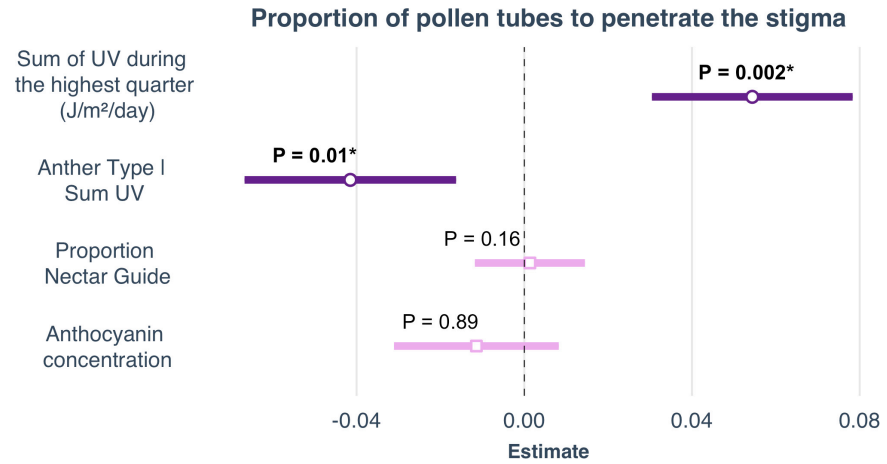


FIGURE 4 | Selected parameter estimates of linear models to determine the effects of climate, latitude and elevation on proportion nectar guide, anthocyanin concentration, petal area, and mean UV petal reflectance. Estimates are visualized as forest plots (using the 'plot_summs' function in the jtools v2.0.1 package). The point within each bar is the estimate of the regression coefficient. The length of the bar represents the 95% confidence interval (CI) of this parameter.

observed in *C. unguiculata* is not consistent with previous research that has shown a significant negative effect of latitude on UV nectar guide in flowers (Koski and Ashman, 2015), but it does suggest that variation in solar UV may contribute to the evolution of anthocyanin production in *C. unguiculata*.

The association between latitude and floral pigment is sometimes associated with underlying climatic variables, such as temperature or precipitation (in addition to, or instead of UV radiation) (Schemske and Bierzychudek, 2001; del Valle et al., 2015; Bontrager and Angert, 2016). For example, in *Linanthus parryae*, periods of heat or drought favor pink- or purple-flowered individuals over white-flowered ones because associated anthocyanins in vegetative tissues enhance stress tolerance (Schemske and Bierzychudek, 2001; Harder and Barrett, 2006). Contrary to the patterns observed in *L. parryae*, among *C. unguiculata* populations, high mean temperature (MTWM) of the field site had no effect on mean anthocyanin concentration. However, as predicted, MAP had a significant negative effect on anthocyanin concentration among populations of *C. unguiculata*.

Elevation (and associated climatic variables) may also be associated with a cline in anthocyanin production (Berardi et al., 2016; Gray et al., 2018). Populations at higher elevations may evolve to produce more flavonoids as a response to increased UV radiation and/or increased herbivore pressure (Nybakken et al., 2004; Gould et al., 2008). For example, Berardi et al. (2016) found that populations of *Silene vulgaris* at higher elevations had more darkly pigmented calyces than those at lower elevations. However, in the current study, elevation had a significant negative effect on estimated anthocyanin concentration in *C. unguiculata*. Populations of this species generally occur at elevations < 1500 m, and the highest elevation population sampled for this study occurred at 1157 m (Lewis, 2012). It is possible that the elevation range of this species is not wide enough to result in local adaptation to elevation alone. Additionally, in some cases, populations at low elevations may be subject to higher levels of herbivory than high elevation sites (Gao et al., 2019), which could explain the relationship between anthocyanin concentration and elevation that we observed.

Petal Area

Several studies of population differentiation have shown that, within species, flowers may be smaller in locations characterized by high temperatures and low water availability (compared to more mesic locations) (Galen, 2000; Bull-Hereñu and Arroyo, 2009; Bontrager and Angert, 2016). This pattern can be explained by the observation that smaller flowers increase female fitness in xeric climates, because they lose less water than large ones (Galen, 2000; Carroll et al., 2001; Herrera, 2005). For example, Galen (2000) found that, under drought conditions, *Polemonium viscosum* (Polemoniaceae) plants with smaller flowers were more likely to survive and produce fruit (compared to plants that produced larger flowers). Consistent with this pattern, Bontrager and Angert (2016) detected a negative effect of historic mean annual temperature on mean petal length among populations of *Clarkia* pulchella. Similarly, Herrera (2005) examined nine populations of *Rosmarinus officinalis* (Lamiaceae) and found that flowers decreased in mass as the habitat became drier and hotter.

In sum, we may expect populations in hot, dry regions to be characterized by small flowers either as a plastic response to reduced resources or as a result of adaptive evolution. Geographic variation in flower size (that is associated with aridity) is of particular interest to evolutionary ecologists because it can give rise to mating system variation (Jonas and Geber, 1999; Runions and Geber, 2000; Button et al., 2012). In the current study, however, MAP and MTWM had no significant effect on petal area among the populations of *C. unguiculata*. Sum UV and latitude both had significant negative effects on petal area, which suggests that solar UV may influence the

evolution of petal area more than temperature and rainfall in this species.

Does Exposure to UVB Light During Floral Development Influence Flower Color, Pattern, or Size?

In addition to our examination of the effects of climate and geography on floral pigment, one of our research goals was to determine whether petal color (i.e., anthocyanin production) responds plastically to UV exposure. In some species, UV radiation has been shown to affect petal color formation (Ben-Tal and King, 1997; Llorens et al., 2015; Henry-Kirk et al., 2018), and petal color in roses intensifies (due to increased accumulation of anthocyanins) following exposure to UV (Maekawa et al., 1980; Henry-Kirk et al., 2018). Anthocyanins are also responsible for the UV-induced coloration of *Anigozanthos* spp. (Haemodoraceae) perianths (Ben-Tal and King, 1997). Furthermore, exposure to UV-B radiation at the pre-bud break stage can affect flower color. For example, a pre-bud treatment that blocked UV exposure decreased anthocyanin production in several species of *Malus* (Rosaceae), resulting in pink petals instead of red ones (Dong et al., 1998). In the current study, our predictions regarding the effects of elevated UVB on floral traits were not corroborated. Plants grown under LED lights (which produce only red and blue light) produced petals with higher estimated anthocyanin concentration than plants grown under LED + UV lights. It is possible that plants within the LED + UV light treatment group produced more non-anthocyanin flavonoids than those in the LED only group. The unexpected increase in anthocyanin production in the LED only group we observed may reflect (unmeasured) differences in non-anthocyanin flavonoids with a shared biochemical pathway. However, we only measured anthocyanin concentration in this study. All plants were grown in greenhouses that block most incoming UV light, although it is possible that the small amount of UV light that did pass through the greenhouse glass was sufficient to stimulate anthocyanin production in this species.

Does UV Nectar Guide Size Serve a Direct Protective Function in This Species?

Previous work suggests that populations growing closer to the equator may produce larger UV-absorbing patterns than those from higher latitude populations (Koski and Ashman, 2015). It has been suggested that this pattern is evidence that Gloger's rule — an ecogeographic "rule" stating that pigmentation of endothermic animals will increase from high-latitude to equatorial regions due to changing selective pressures including heat, humidity, predation and UV irradiance — may apply to plants as well. For example, populations of *Argentina anserina* (Rosaceae) growing closer to the equator are characterized by flowers with larger UV-absorbing bullseyes than populations at higher latitudes (Koski and Ashman, 2015). Koski and Ashman (2015) suggest that this pattern may be a general one in plants. Similar to *A. anserina*, *C. unguiculata* is characterized by within-species variation in UV-absorbing nectar guide size and its wild populations are distributed across a wide latitudinal range. In this study we found the populations of *C. unguiculata* growing closest to the equator are characterized by significantly larger UV-patterns (nectar guides) than those at higher latitudes. However, contrary to our predictions, we found that SumUV had a significant negative effect on proportion nectar guide and nectar guide area. Previous work has shown a significant positive relationship between nectar guide size and pollen receipt in this species (Peach et al., 2020). It is possible that the relationship between latitude and proportion nectar guide observed here reflects adaptation to variation in pollinator availability or assemblage across the state.

Koski and Ashman (2015) confirmed experimentally that high UV may exert direct positive selection on UV pattern size (or the proportion of the petal occupied by the UV-absorbing bullseye) in *A. anserina* by absorbing incoming UV light and preventing it from damaging developing pollen grains. We found no association between UV floral pattern size (of a pollen donor's flower) and pollen performance (PSP and P4.5) under either high or low UV experimental light treatments. *A. anserina* is characterized by flowers that are more bowl-shaped and upward facing than the flowers of *C. unguiculata*. The floral morphology of *A. anserina* may result in more exposure of pollen to direct UV light and therefore stronger selection for floral features that offer pollen protection. Additionally, it is possible the UV nectar guides produced by *C. unguiculata* are not large enough to influence the UV environment of the flower. We conclude that this particular function of UV floral patterns may be restricted to species that grow closer to the equator than *C. unguiculata* or that have more reliably upward facing, or cup-shaped flowers.

We found that plants grown from seeds collected from high UV locations produced pollen that performed better than the pollen produced by plants from low UV locations. While UV floral patterns may not confer any direct fitness advantage (in terms of pollen protection) it appears that high UV populations may be characterized by plants that produce higher quality pollen anyway. The pollen of *C. unguiculata* is often itself pigmented (the pollen produced by the longer set of anthers is typically red-purple). Future studies should examine anthocyanin concentration of the anthers and pollen grains themselves to determine if these pigments offer a direct protective function in high UV environments (Koski and Galloway, 2018). The results presented here also corroborate previous reports of significant differences in pollen performance between the dimorphic anthers of *C. unguiculata* (Peach and Mazer, 2019).

CONCLUSION

This study fills several gaps in our understanding of geographic patterns of floral color, pattern, and size. By measuring multiple components of floral pigment and pattern, we identified a more complex geographic pattern than we would have by examining UV floral pattern or anthocyanin concentration alone. Additionally, we examined the effects long-term climate normals

on population mean genotype, rather than using latitude or elevation as a proxy for local climatic conditions, which improved our ability to detect the effects of specific climatic variables on our focal floral traits. We found that mean proportion nectar guide (the proportion of the petal occupied by the UV-absorbing nectar guide) was highest in low latitude, low-UV populations, which only partially supports our expectations, given the large-scale latitudinal patterns previously reported in *Argentina anserina* (Koski and Ashman, 2015). Mean floral anthocyanin concentration was highest in populations with high UV and low precipitation, which supports our predictions (del Valle et al., 2015). Anthocyanin concentration and proportion nectar guide did not influence pollen performance in either of the light treatments used in this study, indicating that these floral features did not provide a protective function for pollen germination or growth under the conditions examined here.

Abiotic factors contribute to floral diversity and broad-scale geographic patterns of floral color, pattern, and size (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Coberly and Rausher, 2003; Arista et al., 2013; Koski and Ashman, 2015; Tripp et al., 2018) and identifying meaningful signatures of selection on flower color across large-scale environmental gradients is most informative when considering numerous interacting features of climate and topography.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KP and SM conceived the idea, designed the methods and statistical analyses, and led the writing of the manuscript and the interpretation of the results. KP and JL collected the data, participated in analytical discussions, and analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00847/full#supplementary-material

REFERENCES

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Arnold, S. E. J., Savolainen, V., and Chittka, L. (2009). Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. *Arthropod. Plant Interact.* 3, 27–43. doi:10.1007/s11829-009-9056-9

Beckmann, M., Václavík, T., Manceur, A. M., Šprtová, L., von Wehrden, H., Welk, E., et al. (2014). gl UV: a global UV-B radiation data set for macroecological studies. *Methods Ecol. Evol.* 5, 372–383. doi: 10.1111/2041-210X.12168

Ben-Tal, Y., and King, R. W. (1997). Environmental factors involved in colouration of flowers of Kangaroo Paw. *Sci. Hortic.* 72, 35–48. doi: 10.1016/S0304-4238(97)00071-X

Berardi, A. E. (2014). *The Role of the Plant Flavonoid Pathway in Adaptation to Elevation*. Ph.D. dissertation, University of Virginia, Charlottesville.

Berardi, A. E., Hildreth, S. B., Helm, R. F., Winkel, B. S. J., and Smith, S. D. (2016). Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae). *Phytochemistry* 130, 119–127. doi: 10.1016/j.phytochem.2016.05.007

Bergamo, P. J., Telles, F. J., Arnold, S. E. J., and Garcia de Brito, V. L. (2018). Flower colour within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-5

Bontrager, M., and Angert, A. L. (2016). Effects of range-wide variation in climate and isolation on floral traits and reproductive output of *Clarkia pulchella*. *Am. J. Bot.* 103, 10–21. doi: 10.3732/ajb.1500091

Bowman, R. N. (1987). Cryptic self-incompatibility and the breeding system of Clarkia unguiculata (Onagraceae). *Am. J. Bot.* 74, 471–476. doi: 10.1002/j.1537-2197.1987.tb08667.x

Brunet, J. (2009). Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations with floral traits. *Ann. Bot.* 103, 1567–1578. doi: 10.1093/aob/mcp096

Bull-Hereñu, K., and Arroyo, M. T. K. (2009). Phenological and morphological differentiation in annual *Chaetanthera moenchioides* (Asteraceae) over an aridity gradient. *Plant Syst. Evol.* 278, 159–167. doi: 10.1007/s00606-008-0126-8

Burns, K. C. (2015). The color of plant reproduction: macroecological trade-offs between biotic signaling and abiotic tolerance. *Front. Ecol. Evol.* 3:118. doi: 10.3389/fevo.2015.00118

Button, L., Villalobos, A., Dart, S., and Eckert, C. (2012). Reduced petal size and color associated with transitions from outcrossing to selfing in *Camissoniopsis cheiranthifolia* (Onagraceae). *Int. J. Plant Sci.* 173, 251–260. doi: 10.1086/663972

Campbell, D. R., and Powers, J. M. (2015). Natural selection on floral morphology can be influenced by climate. *Proc. R. Soc.* 282:20150178. doi: 10.1098/rspb.2015.0178

Carroll, A. B., Pallardy, S. G., and Galen, C. (2001). Drought stress, plant water status, and floral trait expression in fireweed, *Epilobium angustifolium* (Onagraceae). *Am. J. Bot.* 88, 438–446. doi: 10.2307/2657108

Chittka, L., Thomson, J. D., and Waser, N. M. (1999). Flower Constancy, Insect Psychology, and Plant Evolution. *Naturwissenshaften* 86, 361–377. doi: 10.1007/s001140050636

Christiansen, D. E., Markstrom, S. L., and Hay, L. E. (2011). Impacts of climate change on the growing season in the United States. *Earth Interact.* 15, 1–17. doi: 10.1175/2011EI376.1

Coberly, L. C., and Rausher, M. D. (2003). Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Mol. Ecol.* 12, 1113–1124. doi: 10.1046/j.1365-294X.2003.01786.x

del Valle, J. C., Buide, M. L., Casimiro-Soriguer, I., Whittall, J. B., and Narbona, E. (2015). On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (*Silene littorea*). *Front. Plant Sci.* 6:939. doi: 10.3389/fpls.2015.00939

Del Valle, J. C., Gallardo-López, A., Buide, M. L., Whittall, J. B., and Narbona, E. (2018). Digital photography provides a fast, reliable, and noninvasive method to estimate anthocyanin pigment concentration in reproductive and vegetative plant tissues. *Ecol. Evol.* 8, 3064–3076. doi: 10.1002/ece3.3804

Dong, Y. H., Beuning, L., Davies, K., Mitra, D., Morris, B., and Koostra, A. (1998). Expression of pigmentation genes and photo-regulation of anthocyanin biosynthesis in developing Royal Gala apple flowers. *Funct. Plant Biol.* 25, 245–252. doi: 10.1071/PP97108

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Garcia, J. E., Shrestha, M., and Lunau, K. (2015). Seeing in colour: A hundred years of studies on bee vision since the work of the Nobel laureate Karl Von Frisch. *Proc. Royal Soc. Vict.* 127, 66–72. doi: 10.1071/RS15006

Galen, C. (2000). High and dry: drought stress, sex-allocation trade-offs, and selection on flower size in the alpine wildflower *Polemonium viscosum* (Polemoniaceae). *Am. Nat.* 156, 72–83. doi: 10.1086/303373

Gao, J., Fang, C., and Zhao, B. (2019). The latitudinal herbivory hypothesis revisited: to be part is to be whole. *Ecol. Evol.* 9, 3681–3688. doi: 10.1002/ece3.2759

Garcia, J. E., Greentree, A. D., Shrestha, M., Dorin, A., and Dyer, A. G. (2014). Flower colours through the lens: quantitative measurement with visible and ultraviolet digital photography. *PLoS One* 9:e96646. doi: 10.1371/journal.pone.0096646

Gómez, J., Ramo, C., Stevens, M., Liñán-Cembrano, G., Rendón, M. A., Troscianko, J. T., et al. (2018). Latitudinal variation in biophysical characteristics of avian eggshells to cope with differential effects of solar radiation. *Ecol. Evol.* 8, 8019–8029. doi: 10.1002/ece3.4335

Gould, K., Davies, K. M., and Winefield, C. (eds) (2008). *Anthocyanins: Biosynthesis, Functions, and Applications*. Cham: Springer.

Gould, K. S. (2004). Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *BioMed Res. Int.* 5, 314–320. doi: 10.1155/S1110724304406147

Gray, M., Stansberry, M. J., Lynn, J. S., Williams, C. F., White, T. E., and Whitney, K. D. (2018). Consistent shifts in pollinator-relevant floral coloration along Rocky Mountain elevation gradients. *J. Ecol.* 106, 1910–1924. doi: 10.1016/j.ympev.2018.07.004

Grossenbacher, D. L., and Stanton, M. L. (2014). Pollinator-mediated competition influences selection for flower-color displacement in sympatric monkeyflowers. *Am. J. Bot.* 101, 1915–1924. doi: 10.3732/ajb.1400204

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Hanley, M. E., Lamont, B. B., and Armbruster, W. S. (2009). Pollination and plant defence traits co-vary in Western Australian Hakeas. *New Phytol.* 182, 251–260. doi: 10.1111/j.1469-8137.2008.02709.x

Harder, L. D., and Barrett, S. C. H. (2006). *Ecology and Evolution of Flowers*. Oxford: Oxford University Press.

Hårdh, K., and Hårdh, J. E. (1977). Studies on quality of vegetables and strawberries at different latitudes in Finland. *Ann. Agric. Fenniae* 16, 19–26.

Hawkes, M. F., Duffy, E., Joag, R., Skeats, A., Radwan, J., Wedell, N., et al. (2019). Sexual selection drives the evolution of male wing interference patterns. *Proc. R. Soc. B Biol. Sci.* 286:20182850. doi: 10.1098/rspb.2018.2850

Hedhly, A., Hormaza, J. I., and Herrero, M. (2009). Global warming and sexual plant reproduction. *Trends Plant Sci.* 14, 30–36. doi: 10.1016/j.tplants.2008.11.001

Henry-Kirk, R. A., Plunkett, B., Hall, M., McGhie, T., Allan, A. C., Wargent, J. J., et al. (2018). Solar UV light regulates flavonoid metabolism in apple (*Malus* x *domestica*). *Plant Cell Environ.* 41, 675–688. doi: 10.1111/pce.13125

Herrera, J. (2005). Flower size variation in *Rosmarinus officinalis*: individuals, populations and habitats. *Ann. Bot.* 95, 431–437. doi: 10.1093/aob/mci041

Hoffmann, A. A., and Sgrò, C. M. (2011). Climate change and evolutionary adaptation. *Nature* 470, 479–485. doi: 10.1038/nature09670

Hopkins, R., and Rausher, M. D. (2014). The cost of reinforcement: selection on flower color in allopatric populations of *Phlox drummondii*. *Am. Nat.* 183, 693–710. doi: 10.5061/dryad.f710n

Hove, A. A., and Mazer, S. J. (2013). Pollen performance in *Clarkia* taxa with contrasting mating systems: implications for male gametophytic evolution in selfers and outcrossers. *Plants* 2, 248–278. doi: 10.3390/plants2020248

IPCC AR4, (2007). "Climate change 2007: synthesis report," in *Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds Core Writing Team, R. K. Pachauri, and A. Reisinger (Geneva: IPCC).

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-9658(2003)084[1733:trohit]2.0.co;2

Ivey, C. T., Dudley, L. S., Hove, A. A., Emms, S. K., and Mazer, S. J. (2016). Outcrossing and photosynthetic rates vary independently within two *Clarkia* species: implications for the joint evolution of drought escape physiology and mating system. *Ann. Bot.* 118, 897–905. doi: 10.1093/aob/mcw134

Jaakola, L., and Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in plants. *Plant Cell Environ.* 33, 1239–1247. doi: 10.1111/j.1365-3040.2010.02154.x

Jonas, C. S., and Geber, M. A. (1999). Variation among populations of *Clarkia unguiculata* (Onagraceae) along altitudinal and latitudinal gradients. *Am. J. Bot.* 86, 333–343. doi: 10.2307/2656755

Kay, Q. O. N., Daoud, H. S., and Stirton, C. H. (1981). Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.* 83, 57–83. doi: 10.1111/j.1095-8339.1981.tb00129.x)

Körner, C. (2007). The use of 'altitude' in ecological research. *Trends Ecol. Evol.* 22, 569–574. doi: 10.1016/j.tree.2007.09.006

Koski, M. H., and Ashman, T. L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct. Ecol.* 28, 868–877. doi: 10.1111/1365-2435.12242

Koski, M. H., and Ashman, T. L. (2015). Floral pigmentation patterns provide an example of Gloger's rule in plants. *Nat. Plants* 1:14007. doi: 10.1038/nplants.2014.7

Koski, M. H., and Ashman, T. L. (2016). Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors. *New Phytol.* 211, 708–718. doi: 10.1111/nph.13921

Koski, M. H., and Galloway, L. F. (2018). Geographic variation in pollen color is associated with temperature stress. *New Phytol.* 218, 370–379. doi: 10.1111/nph.14961

Lacey, E. P., Lovin, M. E., Richter, S. J., and Herington, D. A. (2010). Floral reflectance, color, and thermoregulation: what really explains geographic variation in thermal acclimation ability of ectotherms? *Am. Nat.* 175, 335–349. doi: 10.1086/650442

Landi, M., Tattini, M., and Gould, K. S. (2015). Multiple functional roles of anthocyanins in plant-environment interactions. *Environ. Exp. Bot.* 119, 4–17. doi: 10.1016/j.envexpbot.2015.05.012

Lätti, A. K., Riihinen, K. R., and Kainulainen, P. (2008). Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *J. Agric. Food Chem.* 56, 190–196. doi: 10.1021/jf072857m

Lee, D., and Gould, K. (2002). Why leaves turn red. *Am. Sci.* 90:524. doi: 10.1511/2002.6.524

Lee, J., and Finn, C. E. (2007). Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *J. Sci. Food Agric.* 87, 2665–2675. doi: 10.1002/jsfa.3029

Levelt, P. F., van den Oord, G. H., Dobber, M. R., Malkki, A., Visser, H., de Vries, J., et al. (2006). The ozone monitoring instrument. *IEEE Trans. Geosci. Remote Sens.* 44, 1093–1101. doi: 10.1109/TGRS.2006.872333

Lewis, H. (2012). "Clarkia unguiculata," in *Jepson Flora Project*, ed. J. E. Flora. Available at: http://ucjeps.berkeley.edu/eflora/eflora_display.php?tid=19605 (accessed October 24, 2019).

Lewis, H., and Lewis, M. E. (1955). *The Genus Clarkia*. Los Angeles: University of California Press.

Llorens, L., Badenes-Pérez, F. R., Julkunen-Tiitto, R., Zidorn, C., Fereres, A., and Jansen, M. A. K. (2015). The role of UV-B radiation in plant sexual reproduction. *Perspect. Plant Ecol. Evol. Syst.* 17, 243–254. doi: 10.1016/j.ppees.2015.03.001

Lüdecke, D. (2019). *sjstats: Statistical Functions for Regression Models (Version 0.17.7)*.

Macfarlane, C., and Ogden, G. N. (2012). Automated estimation of foliage cover in forest understorey from digital nadir images. *Methods Ecol. Evol.* 3, 405–415. doi: 10.1111/j.2041-210X.2011.00151.x

MacSwain, J. W., Raven, P. H., and Thorp, R. W. (1973). Comparative behaviour of bees and Onagraceae. IV. Clarkia bees of the western United States. University of California Publications: Entomology, 70, 1–80.

Maekawa, S., Terabun, M., and Nakamura, N. (1980). Effects of ultraviolet and visible light on flower pigmentation of 'Ehigasa' roses. *J. Jpn Soc. Hortic. Sci.* 49, 251–259. doi: 10.2503/jjshs.49.251

Martin, F. W. (1959). Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technol.* 34, 125–128. doi: 10.3109/10520295909114663

Mazer, S. J., Hendrickson, B. T., Chellew, J. P., Kim, L. J., Liu, J. W., Shu, J., et al. (2018). Divergence in pollen performance between *Clarkia* sister species with contrasting mating systems supports predictions of sexual selection. *Evolution* 72, 453–472. doi: 10.1111/evo.13429

Mazer, S. J., Moghaddasi, A., Bello, A. K., and Hove, A. A. (2016). Winning in style: longer styles receive more pollen, but style length does not affect pollen attrition in wild *Clarkia populations*. *Am. J. Bot.* 103, 408–422. doi: 10.3732/ajb.1500192

Menzies, I. J., Youard, L. W., Lord, J. M., Carpenter, K. L., Klink, J. W., Perry, N. B., et al. (2016). Leaf colour polymorphisms: a balance between plant defence and photosynthesis. *J. Ecol.* 104, 104–113. doi: 10.1111/1365-2745.12494

Moeller, D. A., and Geber, M. A. (2005). Ecological context of the evolution of self-pollination in *Clarkia xantiana*: population size, plant communities, and reproductive assurance. *Evolution* 59, 786–799. doi: 10.1554/04-656

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Nybakken, L., Aubert, S., and Bilger, W. (2004). Epidermal UV-screening of arctic and alpine plants along a latitudinal gradient in Europe. *Polar Biol.* 27, 391–398. doi: 10.1007/s00300-004-0601-9

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Peach, K., Liu, J. W., Klitgaard, K. N., and Mazer, S. J. (2020). Sex-specific floral attraction traits in a sequentially hermaphroditic species. *Ecol. Evol.* 00, 1–20. doi: 10.1002/ece3.5987

Peach, K., and Mazer, S. J. (2019). Heteranthery in *Clarkia*: pollen performance of dimorphic anthers contradicts expectations. *Am. J. Bot.* 106, 598–603. doi: 10.1002/ajb2.1262

Price, N., Green, S., Troscianko, J., Tregenza, T., and Stevens, M. (2019). Background matching and disruptive coloration as habitat-specific strategies for camouflage. *Sci. Rep.* 9:7840.

Rausher, M. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Runions, J., and Geber, M. A. (2000). Evolution of the self-pollinating flower in Clarkia xantiana (Onagraceae). I. Size and Development of floral organs. *Am. J. Bot.* 87, 1439–1451. doi: 10.2307/2656870

Schemske, D. W., and Bierzychudek, P. (2001). Evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* 55, 1269–1282. doi: 10.1111/j.0014-3820.2001.tb00650.x

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Skorupski, P., and Chittka, L. (2010). Photoreceptor spectral sensitivity in the Bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). *PLoS One* 5:e12049. doi: 10.1371/journal.pone.0012049

Sobral, M., Veiga, T., Domínguez, P., Guitián, J. A., Guitián, P., and Guitián, J. M. (2015). Selective pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS One* 10:e0132522. doi: 10.1371/journal.pone.0132522

Soltis, P. S. (1986). Anthocyanin variation in *Clarkia* (Onagraceae). *Biochem. Syst. Ecol.* 14, 487–489. doi: 10.1016/0305-1978(86)90007-4

Spottiswoode, C. N., and Stevens, M. (2010). Visual modeling shows that avian host parents use multiple visual cues in rejecting parasitic eggs. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8672–8676. doi: 10.1073/pnas.0910486107

Stevens, M., Párraga, A. C., Cuthill, I. C., Partridge, J. C., and Troscianko, J. (2007). Using digital photography to study animal coloration. *Biol. J. Linn. Soc.* 90, 211–237. doi: 10.1111/j.1095-8312.2007.00725.x

Stevens, M., Troscianko, J., Wilson-Aggarwal, J. K., and Spottiswoode, C. N. (2017). Improvement of individual camouflage through background choice in ground-nesting birds. *Nat. Ecol. Evol.* 1:1325. doi: 10.1038/s41559-017-0256-x

Strauss, S. Y., and Cacho, N. I. (2013). Nowhere to run, nowhere to hide: the importance of enemies and apparency in adaptation to harsh soil environments. *Am. Nat.* 182, E1–E14. doi: 10.1086/670754

Streisfeld, M. A., and Kohn, J. R. (2005). Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. *Evolution* 59, 2548–2559. doi: 10.1111/j.0014-3820.2005.tb00968.x

Šulc, M., Troscianko, J., Štětková, G., Hughes, A. E., Jelínek, V., Capek, M., et al. (2019). Mimicry cannot explain rejection type in a host–brood parasite system. *Anim. Behav.* 155, 111–118. doi: 10.1016/j.anbehav.2019.05.021

Tanskanen, A., Krotkov, N. A., Herman, J. R., and Arola, A. (2006). Surface ultraviolet irradiance from OMI. Geoscience and remote sensing. *IEEE Trans. Geosci. Remote Sens.* 44, 1267–1271. doi: 10.1109/TGRS.2005.862203

Torregrosa, A., Combs, C., and Peters, J. (2016). GOES-derived fog and low cloud indices for coastal north and central California ecological analyses. *Earth Space Sci.* 3, 46–67. doi: 10.1002/2015EA000119

Tripp, E. A., Zhuang, Y., Schreiber, M., Stone, H., and Berardi, A. E. (2018). Evolutionary and ecological drivers of plant flavonoids across a large latitudinal gradient. *Mol. Phylogenet. Evol.* 128, 147–161. doi: 10.1016/j.ympev.2018.07.004

Troscianko, J., and Stevens, M. (2015). Image calibration and analysis toolbox – a free software suite for objectively measuring reflectance, colour and pattern. *Methods Ecol. Evol.* 6, 1320–1331. doi: 10.1111/2041-210x.12439

Tsuchimatsu, T., Yoshitake, H., and Ito, M. (2014). Herbivore pressure by weevils associated with flower color polymorphism in *Geranium thunbergii* (Geraniaceae). *J. Plant Res.* 127, 265–273. doi: 10.1007/s10265-013-0598-7

van den Berg, C. P., Troscianko, J., Endler, J. A., Marshall, N. J., and Cheney, K. L. (2019). Quantitative colour pattern analysis (QCPA): a comprehensive framework for the analysis of colour patterns in nature. *Methods Ecol. Evol.* 11, 316–332. doi: 10.1111/2041-210X.13328

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B* 283:20160429. doi: 10.1098/rspb.2016.0429

van der Niet, T., and Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol. Evol.* 27, 353–361. doi: 10.1016/j.tree.2012.02.002

Wang, T., Hamann, A., Spittlehouse, D. L., and Carroll, C. (2016). Locally downscaled and spatially customizable climate data for historical and future periods for North America. *PLoS One* 11:e0156720. doi: 10.1371/journal.pone.0156720

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Whittall, J. B., and Strauss, S. Y. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder, and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology and biotechnology. *Plant Physiol.* 126, 485–493. doi: 10.1104/pp.126.2.485

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Macroevolution of Flower Color Patterning: Biased Transition Rates and Correlated Evolution with Flower Size

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Floral pigmentation patterns can both mediate plant-pollinator interactions and modify the abiotic environment of reproductive structures. To date, there have been no inquiries into the rate and directionality of macroevolutionary transitions between patterned and non-patterned petals despite their ecological importance and ubiquity across angiosperms. Petals in the Potentilleae tribe (Rosaceae) display color patterns in the ultraviolet (UV) and human-visible spectrum, or can be uniform in color (i.e., patternless). Using a phylogeny of Potentilleae, I test whether evolutionary transition rates between patterned and non-patterned petals are biased in either direction. I then examine whether UV and human-visible floral patterns are phylogenetically correlated and test the prediction that color patterns will evolve in concert with larger flowers if they function as guides to orient pollinators to floral rewards. I found that transition rates were biased toward petals that were uniform in color. Transition rates from patterned to uniformly colored petals were two and six times higher than the reverse for UV and human-visible pattern, respectively. The presence of UV and human-visible pattern evolved independently from one another. However, the evolution of human-visible pattern was associated with the evolution of larger flowers but the evolution of UV pattern was correlated with the evolution of smaller flowers. I posit that the transition bias toward non-patterned flowers may reflect developmental constraints on spatial regulation of pigments required to produce floral color patterning. The correlated evolution of larger flowers and human-visible pigmentation patterns support the hypothesis that nectar or pollen guides are more likely to evolve in larger-flowered species. This work provides insight into how transition rate bias and trait correlations can shape phylogenetic patterns of floral color pattern diversity.

Keywords: correlated evolution, floral evolution, flower color, flower color pattern, hidden rates model, nectar guide, pollination, transition rate

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INTRODUCTION

Petal color spots, patterns, and lines are common across angiosperms and are important for mediating plant-animal interactions. For example, color patterns on petals can enhance pollinator's ability to detect flowers (Lehrer et al., 1995; Koski and Ashman, 2014), orient to floral rewards (e.g., Manning, 1956), and increase the likelihood of effective pollination (Hansen et al., 2012). Patterning can also discourage nectar robbers (Leonard et al., 2013) and function as deterrents to florivores (Gronquist et al., 2001). A particularly frequent floral color pattern is one whereby petal bases display different spectral signatures than petal apices, manifesting a bulleseye or target (e.g., Penny, 1983; Hempel de Ibarra and Vorobyev, 2009; Hempel de Ibarra et al., 2015; **Figure 1**). These "bull's-eye" patterns are common in both the human-visible and ultraviolet (UV) spectrum (McCrea and Levy, 1983). While UV color patterns influence pollinator choice and behavior (e.g., Koski and Ashman, 2014; Peterson et al., 2015; Brock et al., 2016; Papiorek et al., 2016), there is also support for the role of UV patterns in protecting pollen from abiotic stress (Koski and Ashman, 2015a). Despite the ubiquity of color patterns and their myriad ecological roles in plant reproduction, we understand little about their evolutionary history.

Many studies have dissected the biochemistry and development of flower color patterning but none have evaluated their macroevolutionary dynamics. In fact, there are few studies on the tempo and directionality of macroevolution for ecologically relevant floral traits in general (however, see Smith and Goldberg, 2015; Wessinger et al., 2019 for pigment presence/absence; and Ree and Donoghue, 1999 for symmetry). Bull's-eye pigmentation patterns can be manifested by the spatial regulation of pigments in multiple ways. First, pigments can accumulate at the base of petals but be absent at the tips (e.g., Harborne and Nash, 1984; Jorgensen, 1995). Alternatively, pigments can be restricted to petal tips but absent at the bases (e.g., Jorgensen, 1995). Thus, the absence of petal patterning could result from either the uniform production of a pigment across the petal or the loss of the pigment altogether. In either case, it is unknown whether evolutionary transitions between patterned and non-patterned flowers are biased in either direction. Determining transition rates in color patterns across a clade with both patterned and non-patterned flowers will give insight into whether there is asymmetry in evolutionary transitions between the character states. For example, an evaluation of the tempo and mode of floral color evolution across multiple clades revealed that gains in pigmentation were more common than losses (Smith and Goldberg, 2015).

The ecological functions of floral patterns, and correlations with other floral traits have the potential to influence their phylogenetic distributions. Experiments with natural and artificial flowers support that petal patterns influence pollinator alighting and orienting behaviors, but petal patterning may be a more salient cue for pollinators in larger flowers (Lawson and Rands, 2018). Bull's-eye patterns increase the ability of pollinators to orient to the center of flowers (Manning, 1956; Free, 1970; Johnson and Dafni, 1998; Dinkel and Lunau, 2001; Lunau et al., 2009) and reduce handling time by pollinators

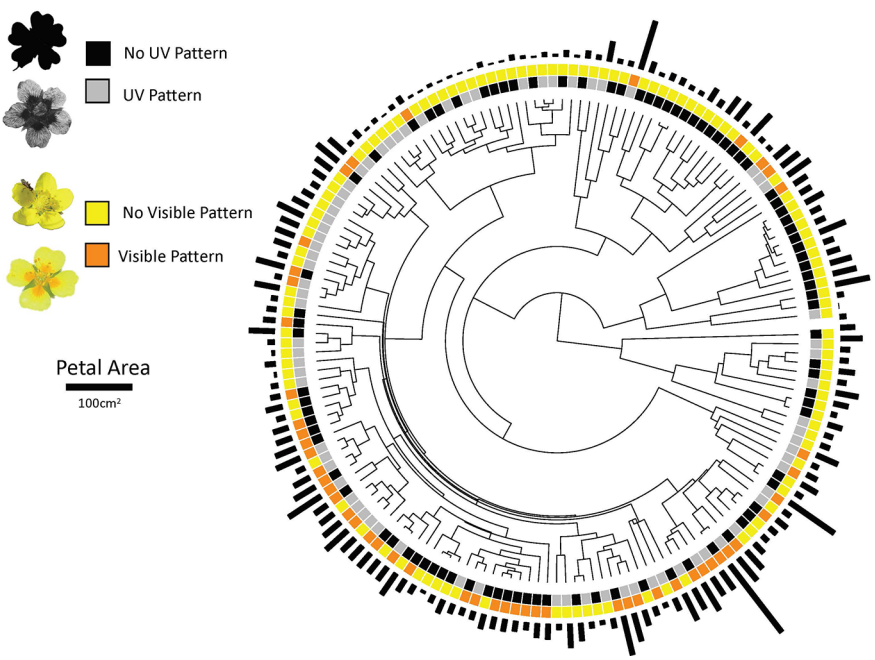


FIGURE 1 | The phylogenetic distribution of ultraviolet and human-visible floral pigmentation patterns in and petal area in the Potentilleae tribe. Examples of species that lack UV pattern (*Potentilla evestita*), have UV pattern (*P. eriocarpa*), lack visible pattern (*Argentina anserina*), and have visible pattern (*P. erecta*) are provided in the key.

(Waser and Price, 1985; Leonard and Papaj, 2011; de Jager et al., 2017). However, Koski and Ashman (2014) showed that in small radially symmetric flowers (~15 mm diam.), the presence of floral patterning failed to enhance the ability of small solitary bees and syrphid files to land on the center of flowers or orient to floral rewards after landing. Patterns that fail to enhance landing or orientation however may enhance contact between pollinators and anthers (see Dinkel and Lunau, 2001). Conversely, Kelber (1997) found that hawkmoths alighted to the periphery of large flower models lacking pattern (64 mm diam.). Likewise, Lunau et al. (2006) demonstrated that bumblebees approach petal peripheries in the absence of central color spots, again when the floral models were large (> 67 mm diam.). Together, these studies suggest that petal patterns may be more important in orienting pollinators in larger flowers. Additionally, a genetic algorithm that accounted for pollinator behavior predicted that larger flowers would be more likely to develop floral guides (Lawson and Rands, 2018). If pollinator-mediated selection drives the evolution of bull's-eye patterns, we may expect a positive phylogenetic correlation between flower size and the presence of floral patterning.

Some surveys of petal color patterning in flowering plant communities support that patterning is more common in larger-flowered species. A survey of UV floral pattern in 300 Californian taxa found that the presence of pattern increased with flower size (Guldberg and Atsatt, 1975). This finding was corroborated in a broad survey of the Hawaiian flora (Jones et al., 1999). However, Dyer (1996) found that intermediate-sized flowers were more likely to possess UV-reflection in the Australian flora. A comparative lens using a group of species with and without petal pattern is one way to shed light on the hypothesis that patterns will be more likely to evolve in larger-flowered species.

In the Potentilleae, flowers can display human-visible (Asker, 1985; **Figure 1**) or UV bull's-eye patterns (Koski and Ashman, 2016; **Figure 1**). UV petal patterning is caused by the production of various UV-absorbing flavonol compounds present at the base of petals but absent at the tips of petals (Harborne and Nash, 1984). Petals are often uniformly pigmented by carotenoids which do not strongly absorb UV (Goodwin, 1980). Thus, flowers that are uniformly UV-absorbing produce flavonols throughout the entire petal. Across nearly 200 Potentilleae species, roughly 50% are uniformly UV-absorbing while the other half are patterned (Koski and Ashman, 2016; **Figure 1**), making it an ideal group for macroevolutionary studies of floral color patterns. Human-visible bull's-eye patterns are also present in Potentilleae due to dark yellow to orange pigmentation at the base of petals with brighter yellow petal apices (**Figure 1**). The biochemistry of visible patterning has not yet been illuminated in Potentilleae, however pigments underlying human-visible patterning are likely carotenoid compounds that are restricted to petal bases. Since UV and human-visible patterns are relevant to pollinator visual systems (Briscoe and Chittka, 2001), expression of both patterns which utilize different biochemical pathways could be functionally redundant with respect to mediating plant-pollinator interactions. If that is the case, visible patterns and UV patterns may display negative phylogenetic correlation such that visible patterns are more likely to evolve in species that uniformly absorb UV, and UV patterns would be more likely to evolve in flowers that lack visible pattern.

Using a phylogeny of Potentilleae, I characterize the presence and absence petal patterning in the UV and human-visible spectrum, as well as flower size to answer the following questions: 1) Are transitions between patterned and patternless flowers uniform or biased in one direction? 2) Are the evolution of UV and human-visible petal patterning correlated? 3) Are bull's-eye patterns more likely to evolve in larger flowers?

METHODS

System

Potentilleae is a globally distributed group of an estimated ~500 taxa. Flowers are all radially symmetrical, and the majority are yellow, though white and red/pink flowers have evolved. Flowers are highly generalized with respect to pollination, being pollinated by various fly species, solitary bees, and in some cases butterflies. One study revealed that both flies and solitary bees effectively transfer pollen (Koski and Ashman, 2015b). Harborne and Nash (1974) characterized the biochemical properties of flowers in 26 Potentilleae taxa; 16 with UV bull's-eye patterns and 10 without. Various flavone, flavonone, and flavonol compounds were responsible for UV absorption in both patterned and non-patterned flowers. Carotenoids do not absorb UV, and have been identified as the yellow pigments in multiple *Potentilla* species (Goodwin, 1980). Basal petal pigmentation spots or bull's-eye apparent in the human visible spectrum have not been characterized biochemically in Potentillae. However, they are often dark yellow to orange which is characteristic of carotenoid pigmentation (Goodwin, 1980). In one species with human-visible pattern (*Potentilla recta*), epidermal petal peels indicate that darker petal bases are caused by carotenoid pigmentation (**Supplemental Figure S1**).

Scoring Petal Pattern and Flower Size

UV pattern and petal area were scored by photographing pressed flowers on an average of 3.2 herbarium specimens per 177 species (see Koski and Ashman, 2015). Petal area was scored as the area of one petal per flower in mm^2. Species were considered patterned in the UV spectrum if a the average proportional petal area that absorbed UV was ≤95%. Two taxa were uniformly UV-reflecting but binned into the UV pattern character state. Seven taxa were 90% to 95% absorbing but were binned into the non-patterned character state. Petal pattern in the human-visible spectrum could not be scored from herbarium records because human-visible pigments often degrade in pressed flowers (Koski, pers. obs.). The presence or absence of human-visible petal patterns were obtained using images from online databases (iNaturalist, Calflora, GBIF, USDA Plants) or descriptions from online flora (e.g., Flora North America, Flora of China). Visible petal pattern was scored as "present" or "absent." For 13 species, I was unable to obtain data on visible petal patterning

from online sources. For any comparative model that contained human-visible pattern, the data set and phylogeny were truncated to 164 species that had data for all three traits scores; UV pattern, human-visible pattern, and petal size. For taxa that possessed both UV and human-visible petal patterning, I could not determine whether basal petal spots were congruent (i.e., completely overlapping) because UV images were obtained from herbarium specimens while human-visible pattern data was obtained from online images. Additionally, because human-visible pattern was obtained from standard human-visible images, the presence of "blue bull's-eye's" that may be perceived by pollinators (e.g., Verhoeven et al., 2018) but not humans, were not considered. Finally, I only considered petal patterning but not floral patterns caused by contrasting reproductive structures (e.g., Lunau, 2006).

Phylogeny

For comparative analyses I used the time-calibrated Bayesian phylogeny of 183 Potentilleae generated by Koski and Ashman (2016) by combining two nuclear (ITS, ETS) and one chloroplast (trnLF) marker. For the current study, 200 phylogenies from the posterior distribution were trimmed to represent the species for which UV pattern, human-visible pattern, and petal size data could be obtained. All phylogenetic analyses were conducted on 200 phylogenies from the posterior distribution to account for phylogenetic uncertainty. For analyses that included UV pigmentation pattern and petal size, phylogenies were trimmed to 177 species. For analyses that included visible pigmentation pattern, phylogenies were trimmed to 164 species.

Evolutionary Transition Rates Between Patterned and Patternless Flowers

To estimate transition rates between patterned and non-patterned flowers in both the UV and human-visible spectrum, I used hidden rate models (Beaulieu et al., 2013). Hidden rate models allow for variation in transition rates among lineages and account for the effects of unmeasured correlates of character states on transition rates. I used the hidden rate model for two primary reasons. First, for phylogenies that are large in taxonomic and geographic scope (e.g., the Potentilleae phylogeny used here samples from 5 genera with a global distribution), homogeneity in evolutionary rates across lineages is less likely (Beaulieu et al., 2013). Second, the hidden rate model may also account for the effects of unmeasured traits or environmental factors on evolution of the focal trait (Beaulieu et al., 2013).

I tested three transition rate models for each patterning trait: 1) a model with no hidden transition rates, 2) a model with one hidden rate allowing for "fast" and "slow" transitions, and 3) a model with two hidden rates allowing for "fast," "medium," and "slow" transitions (see Rivkin et al., 2016). Each of the six models (3 for UV pattern, 3 for human-visible pattern) were performed on 200 phylogenetic trees from the posterior distribution. For the model of UV patterning, 10 random starts were used per model optimization. However, for visible pattern, 5 random starts were used due to constraints on computational resources. I compared model fits between models 1 and 2, and models 2 and 3, to determine whether the addition of a hidden rate improved model fit. Model comparison was performed using a log-likelihood ratio tests. Average transition rates of the best-fit model across all 200 trees are presented. The marginal ancestral states were plotted on the Maximum Clade Credibility (MCC) tree generated from a corHMM model with 50 random starts.

Testing for Correlated Evolution

To test whether human-visible pattern was more likely to evolve in flowers that lacked UV patterning, I used Pagel's Test for correlated evolution between visible patterning and the absence of UV patterning (Pagel, 1994). Pagel (1994) test compares model fit parameters between an evolutionary model whereby transitions in one binary trait depend on another binary trait, and a model whereby each trait evolves independently. Using the corDISC function (corHMM package), I fit a model where the transitions between visible pattern and patternless flowers were dependent on transitions between UV-patterned and paternless flowers permitting different transition rates among characters ("ARD" option). I calculated the average log likelihood across all 200 posterior trees and compared it to the summed the log likelihood values from the independent models for UV pattern and human-visible pattern with zero hidden rates using a likelihood ratio test (Rivkin et al., 2016). If the log-likelihood was significantly higher for the dependent model compared to the independent model, I concluded that the evolution of human-visible pattern was correlated with the evolution of UV pattern.

To test whether the evolution of patterned flowers (UV- and human-visible) were associated with the evolution of larger-flowered species, I used generalized estimating equations (Paradis and Claude, 2002; GEE; compar.gee function, "ape" package). Specifically, I modeled the presence (1) and absence (0) of pattern as a function of petal size with a binomial distribution, a logit link function, and the phylogeny with branch lengths transformed to 1 as the correlation structure. I used separate models for the presence/absence of human-visible patterning and the presence/absence of UV patterning. Petal area was log-transformed to achieve normality. GEE models were run on the 200 posterior trees, and model parameters were averaged. The model for human-visible patterning failed to converge on 8 of the 200 posterior trees (4%), thus I report the average parameter values from models using 192 trees. When there was a significant phylogenetic relationship between petal size and petal patterning based on the GEE results, I generated PIC values for each trait using the MCC phylogeny to visualize the relationship.

RESULTS

Floral Color Pattern

Of 177 species in the Potentilleae tribe examined in this study, 48% possessed UV floral patterning (n = 85) while 93 were uniformly UV-absorbing. Fewer species (n = 50 of 164 with data available on visible patterning; 30.5%) had visible petal

patterning while the remaining were uniform in color. For all species with data available for both UV and visible patterning (n = 164), 23 possessed both UV and visible pattern, 52 had UV pattern but lacked visible pattern, 27 were visibly patterned but lacked UV pattern, and 62 displayed neither UV nor visible patterning (**Figure 1**). Thus, the majority of species (~63%) display petal patterning in the visible spectrum, UV spectrum or both.

Transition Rates—UV Patterning

For evolutionary transition rates between UV-patterned and patternless species, a model with one hidden rate was supported (**Table 1**). That is, transition rates between patterned and non-patterned flowers in the UV spectrum varied across the phylogeny between two rates (**Supplemental Figure S1**). The transition rate from uniformly UV-absorbing to UV-patterned flowers was 2.163 while the reverse was 5.323 (**Figure 2A**). Thus, transitions toward uniform UV absorption were about 150% as frequent as the reverse. Transition rates between UV pattern states (patterned fast and patterned slow) were however much higher than transition rates among the two patternless states (9.21 vs 0.001; **Figure 2A**).

TABLE 1 | Model fit comparisons of evolutionary transition rates between patterned and non-patterned petals.

	Number of parameters	Log Likelihood	AIC	χ^2	P
UV color pattern					
No hidden rates	2	–115.109	234.24		
One hidden rate	8	–107.898	231.79	14.422	0.020
Two hidden rates	14	–106.759	241.51	2.278	0.892
Visible color pattern					
No hidden rates	2	–91.4	186.81		
One hidden rate	8	–85.22	186.45	12.36	0.054
Two hidden rates	14	–84.726	197.45	0.988	0.986

The model with no hidden rates assumes that rates do not vary across the phylogeny. The "one hidden rate" model assumes that two rates can vary across the tree and the "two hidden rates" model assumes that three rates can vary across the tree. Chi-square values were generated from log-likelihoods between the more complex model against the simpler model (e.g., two rates vs. one rate) and P values were calculated using a log-likelihood ratio test. All models were run on 200 posterior trees to account for phylogenetic uncertainty and the average log likelihood values were compared.

Ancestral state reconstruction of characters and transition rates show that transitions rates vary across the phylogeny, but within given clades the most likely rate is largely consistent (**Supplemental Figure S1**). For example, uniform UV absorption (slow) dominated in the *Drymocallis* and Alba clades but was rare elsewhere in the phylogeny (**Supplemental Figure S2**). Uniform UV absorption (fast) was frequent in the Ivesiod clade and within some clades within the large *Potentilla* group. UV patterning (slow) was common in numerous clades within the *Potentilla* group and the Reptans clade (**Supplemental Figure S2**). The presence of UV pattern is the most likely character state for the ancestor of the diverse Potentilla group (> 75% likelihood; **Supplemental Figure S2**). UV pattern (fast) is an infrequent ancestral state because transitions away from this state are the most frequent (**Supplemental Figure S2**; **Figure 2A**).

Transition Rates—Visible Patterning

For human-visible patterning, a model with one hidden rate was supported over a model without hidden rates (**Table 1**), though the likelihood-ratio test was only marginally significant (P = 0.054). Therefore, I report results of both the single- and 2-rate model. Given a single rate model (no hidden rates), transitions from patterned to non-patterned flowers were about 80% more frequent than the reverse (0.0216 vs. 0.012; **Figure 2B**). However, under a two-rate model, transitions from patterned to non-patterned flowers were about 6 times as frequent as the reverse (1.145 vs. 0.163; **Figure 2C**). In the model with one hidden rate,

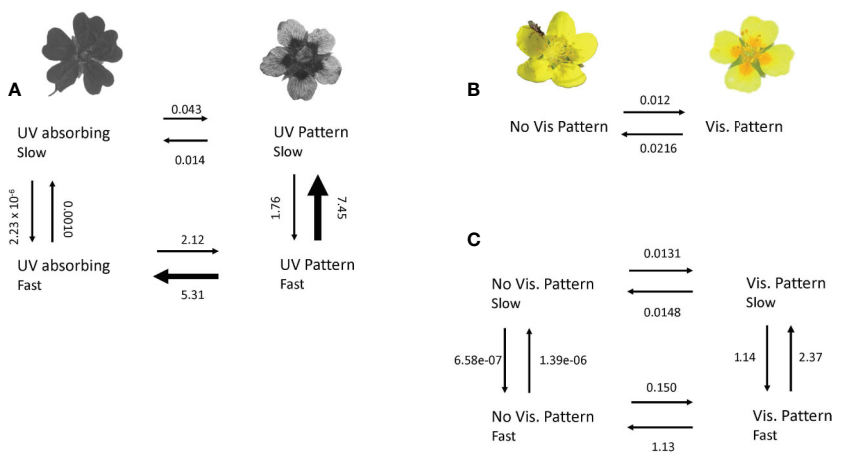


FIGURE 2 | Evolutionary transition rates among patterned and patternless flowers in **(A)** the UV spectrum given the best fit model of a single hidden rate, **(B)** the human-visible spectrum given a model with no hidden rates, and **(C)** the human visible spectrum given a model with a single hidden rate. The single hidden rate model for visible pattern is a marginally better fit than the model without a hidden rate ($P = 0.054$). Arrow widths are proportional to rates. Rates are presented in events per million years.

transitions among patterned states were again much higher than transitions among non-patterned states (3.51 vs. 0.00001; **Figure 2C**).

Ancestral states reconstruction for the model without a hidden rate for visible pattern shows that the presence of visible pattern dominated in the *Potentilla* group but the absence of visible pattern dominated in all clades outside of this group (**Supplemental Figure S2**). There is support for a visibly patterned common ancestor of *Potentilla* clade under models with or without a hidden rate (**Supplemental Figure S3**, **S4**).

Correlated Evolution

Independent models of evolution for UV pattern and visible pattern were better supported than a model of dependent evolution (**Table 2**). That is, UV and visible color patterns evolved independently from one another. This is highlighted by the fact that visible color patterns were not more likely to occur in species that lack UV patterns. Of the 50 taxa that were visibly patterned, 27 (54%) were UV-patterned and 23 (46%) were uniformly UV-absorbing.

TABLE 2 | Pagel (1994) test of correlated evolution for UV- and human-visible petal patterning in Potentilleae.

	Log Likelihood	χ^2	*P*
Independent	−206.509		
Dependent	−203.678	5.662	0.226

*The dependent model assumes that UV- and human-visible pattern evolve independently and the log-likelihood is the sum of log likelihoods from the single rate visible and UV pattern model (see **Table 1**). The dependent model assumes that the evolution of human visible patterning depends on the presence/absence of UV patterning. Log likelihoods were generated from running each model on 200 random posterior trees to account for phylogenetic uncertainty.*

Contrary to the prediction that floral patterns will evolve in concert with larger flowers, the presence of UV pattern was associated with smaller flowers (parameter estimate = −0.409 ± 0.16 SE; T= −2.73, df P = 28.1, *P* = 0.03; **Figure 3A**). On the other hand, the presence of human-visible patterning evolved in concert with the evolution of larger flowers as predicted (Parameter Estimate= 1.570 ± 0.33 SE; T= 4.75, df P = 27.6, *P* = 0.0004, **Figure 3B**). Thus, expectations were met for human-visible, but not UV pattern.

DISCUSSION

Comparative work in the Potentilleae tribe, a group that varies in the presence of petal color patterns, supports that evolution is biased toward the absence of floral patterning for both patterns caused by human-visible pigments and UV-absorbing pigments. Elevated rates of evolution toward non-patterned flowers support that evolution of color patterning is likely more developmentally complex than uniform production of pigments across the entire petal. Visible color patterning was not more likely to evolve in flowers that lacked UV petal patterning, and UV patterning was not more likely to evolve in flowers that lacked visible patterning, suggesting that visible and UV patterning may not be functionally redundant. Visible petal patterns evolved in concert with larger flowers, providing support for the hypothesis that "nectar guides" may be more likely to evolve in larger flowers. Conversely, UV patterns evolved in concert with smaller flowers suggesting that either UV patterns are not pollinator-orienting cues, or that smaller flowers evolve UV patterns to increase pollinator attraction from a distance. This study sheds light on the evolutionary history of petal patterning and sets the stage for studies examining the development and functional roles of floral color patterns in the Potentilleae tribe.

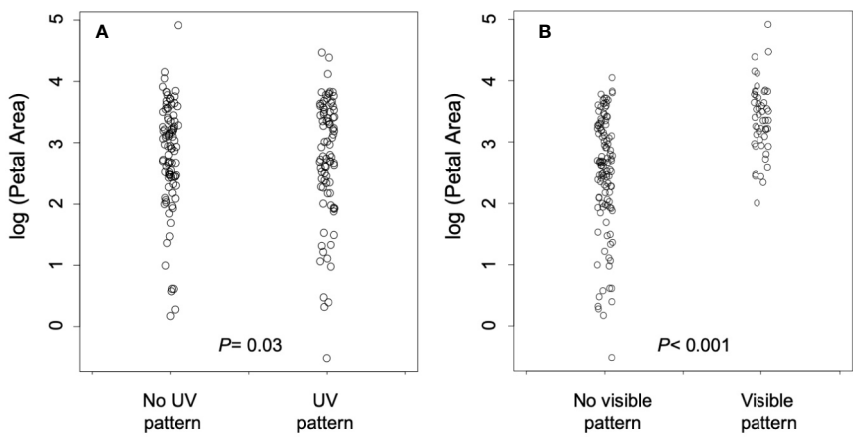


FIGURE 3 | (A) The relationship between petal area and the presence/absence of UV pigmentation pattern. **(B)** The relationship between petal area and the presence/absence of human-visible pigmentation pattern. *P* values in each panel are from phylogenetically controlled generalized estimating equations testing the association between the presence of pattern and flower size.

Biased Transition Rates Toward Patternless Flowers

Assessing the results of transition rate analyses for both UV- and human-visible pattern provides a consistent trend of asymmetric transitions toward color uniformity. Biased evolutionary transitions toward uniform coloration may have developmental or ecological underpinnings. First, evolving pattern often requires precise regulation of the domain of expression of pigments (e.g., *via* alterations to MYB transcription factor expression or their binding sites; Mol et al., 1998; Quattrocchio et al., 2006; Jiang and Rausher, 2018; Ding et al., 2020). Thus, expression of uniform petal coloration may require fewer modifications in fewer structural or regulatory elements of pigmentation pathways compared to the precise spatial regulation of pigment production required for the evolution of color patterning. Additionally, color patterning could be easily lost in a lineage if it does not have a strong selective advantage over uniform coloration. In some systems for instance, selection for increased an increased UV-absorbing area on petals can be strong—Koski and Ashman (2015a) showed that elevated pigmentation protects pollen from UV stress and that across species in the Potentilleae those with ranges in higher-UV environments have elevated pigmentation (Koski and Ashman, 2016).

Past work has characterized the absence of pigmentation as a "loss" and the presence of pigmentation a "gain" (Zufall and Rausher, 2004; Smith and Goldberg, 2015). Treating UV color patterns in a similar binary manner, however, is difficult. The evolution of a uniformly UV-absorbing flower from a UV-patterned flower could be categorized as a gain in pigmentation (pigments are produced at the apex of the petal whereas they were not previously produced in an ancestor). Alternatively, this transition could be viewed as a loss of regulatory ability to restrict pigment production to the bases of petals. Moreover, for either character state, the UV-absorbing pigment is being produced, so the pigment itself is neither lost nor gained. Indeed others have cautioned against categorizing the evolution of a given character state as a loss or gain (Stern and Orgogozo, 2008). Regardless, the biased transition rate toward uniform UV absorption suggests that once petals produce UV-absorbing pigments uniformly across petals, evolving mechanisms to restrict expression to petal bases is rare.

Basal petal spots or patterns in the human-visible spectrum could also be considered a gain or a loss depending on the mechanism by which pigmentation patterns arise. While speculative, the presence of human-visible pigmentation pattern may be underlain by the production of a novel carotenoid that is absent in flowers that lack pattern. If this is the case, then patterning could be considered a gain in pigmentation. Thus, the biased transition rate toward the lack of pigmentation patterns in the human-visible spectrum would suggest that losses are more common than gains which differs from a comparative study on the presence or absence of anthocyanin pigmentation in three groups (Smith and Goldberg, 2015). If evolution toward uniform coloration is caused by the loss of a pigment, then this study is consistent with Dollo's Law (Gould, 1970; Bull and Charnov, 1985) that suggests losses of complex characters are irreversible. The irreversibility of trait loss is expected due to the statistical unlikelihood of re-evolving the exact ancestral phenotype after its loss. For both UV- and human-visible pattern, biochemical studies will be a necessary first step to uncover the developmental basis of petal patterning in Potentilleae. Additionally, pinpointing the ancestral character states of Potentllieae, and estimating the number of evolutionary transitions between states will be important for fully assessing petal patterns in the context of Dollo's Law.

Biased transitions toward color uniformity may have ecological implications with respect to plant-pollinator interactions as well as protection of pollen and ovules from abiotic stress. First, the loss of petal pattern could change the efficiency of foraging by pollinating insects (e.g., Dinkel and Lunau, 2001; Lunau et al., 2006), and if patterns are important to some but not all pollinators, evolutionary transitions to color uniformity could be accompanied by a transition in dominant pollinators. A thorough investigation of pollinator importance across taxa in Potentilleae would be important to examine the role of pollinators in contributing to evolutionary transitions in petal pattern (e.g., Smith, 2010). Larger UV-absorbing areas on petals are associated with increased protection of pollen from UV stress (Koski and Ashman, 2015a), and the evolution of uniform UV absorption tends to be associated with geographic shifts into areas of elevated ambient UV-B irradiance (Koski and Ashman, 2016). Whether evolutionary shifts toward habitats with higher UV-B irradiance are also directional would shed light on how habitat shifts could underlie the biased transition rates.

Some caveats must be considered when interpreting the transition rates reported from the hidden-rates model. First, estimates of species diversity in the Potentilleae are somewhere over 500 species with five to six genera [Angiosperm Phylogeny Group; Steven (2017)]. While the phylogeny sampled 5 genera spanning a broad geographic range, missing taxa could skew results. Second, the transition rates obtained could be confounded by state-dependent diversification (Beaulieu and O'Meara, 2016). For instance, biased transition rates toward a given state could be driven by increased diversification rates of lineages with the given state (Ng and Smith, 2014). While this could be the case for human-visible patterning (~70% of taxa sampled were uniformly colored), I do not expect that biased rates toward uniform UV coloration are driven by an abundance of non-UV-patterned species in the phylogeny of Potentilleae (~50 were uniformly UV-absorbing while half were UV-patterned). Regardless, increased sampling in Potentilleae, increased phylogenetic resolution, and employment of state-dependent models of diversification would help to strengthen the understanding of macroevolutionary transitions in petal color patterning in this group.

Phylogenetic Trait Correlations With the Evolution of Floral Patterning

I predicted that human-visible and UV pattern would display negative phylogenetic covariance if each type of petal pattern were functionally redundant for enhancing pollinator attraction and orientation. That is, visible patterns should evolve when flowers are uniformly absorbing and UV patterns should evolve when

flowers are visibly uniform in color. The fact that this is not the case in Potentilleae suggests that human-visible and UV patterning are unlikely to be functionally redundant as pollinator-orienting cues. Indeed, UV pigmentation protects pollen from UV damage in addition to increasing attractiveness to pollinators (Koski and Ashman, 2014; Koski and Ashman, 2015b), but the ecological role of visible patterning is unknown in this group. From a biochemical standpoint, the lack of covariance between UV- and human-visible patterning may not be surprising given that UV absorption is manifested through flavonoids while darker-orangish petal bases are likely caused by carotenoids. Because pigments underlying human-visible and UV patterning are likely produced by independent pigment pathways, the evolution of human-visible and UV patterning may thus be independent.

The evolution of patterning in the human-visible spectrum and UV spectrum differ in their relationship with the evolution of flower size. While previous work has suggested that across diverse plant communities, the presence UV patterning is associated with larger flowers (Guldberg and Atsatt, 1975), this is not the case when conducing a phylogenetically controlled analysis in Potentilleae. In fact, the evolution of larger flowers was associated with the evolution of uniform UV absorption, contrary to the expectation if UV patterns were pollinator-orienting cues. However, one cannot rule out that the observed pattern could be explained by pollinator mediated selection. If smaller flowers are less attractive to pollinators than larger flowers (Johnson et al., 1995; Conner and Rush, 1996) they may be under selection to evolve additional visual signals to attract pollinators, like UV-reflective patterns (e.g., Hirota et al., 2019). Additionally, if smaller flowers are pollinated by small insects with low-resolution compound eyes, floral guides may still be important for orienting pollinators once they are very close to flowers and are able to distinguish the patterns (Hempel de Ibarra et al., 2015).

There was strong evidence for the correlated evolution between larger flowers and the presence of human-visible color patterning, supporting the prediction that flower color patterns may act as important cues to orient pollinators in larger flowers. For instance in large flowers, central petal spots increase pollinator's ability to alight to the center of flowers (Kelber, 1997; Lunau et al., 2006). This phylogenetic pattern has not yet been documented in other systems, but corroborates two studies showing an increased likelihood of petal color patterning in larger-flowered species in two communities (Guldberg and Atsatt, 1975; Jones et al., 1999) as well as a genetic algorithm predicted that larger flowers are more likely to evolve floral guides (Lawson and Rands, 2018). To my knowledge, behavioral studies that evaluate the efficacy of bull's-eye patterns as attracting and orienting cues for pollinators in flowers of varying size have not been done but will be important for determining the potential driver of this phylogenetic pattern.

CONCLUSIONS

Flowering plants display enormous variation in petal color patterns, and numerous studies have evaluated their ecological significance and development. This is the first study to draw on these ecological and developmental studies to assess how they may shape macroevolutionary patterns and processes for floral color patterns. Developmental constraints may slow the rate of evolution of petal patterning from uniformly colored ancestors leading to bias in evolutionary transition rates. Additionally, intrinsic genetic or ecologically driven correlations between flower size and color patterns are important for shaping the phylogenetic distribution of color patterning. Whether the efficacy of petal patterns as pollinator-orienting cues depends on flower size, and dissection the biochemical and genetic underpinnings of pattern evolution in Potentilleae are underway.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://datadryad.org/stash/share/8EpJTtK7Q3639i44NQy4wC6gAwx2JF9qtDgfQhICMn0.

AUTHOR CONTRIBUTIONS

MK conceived of the study, collected and analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00945/full#supplementary-material

REFERENCES

Asker, S. (1985). Chromosome studies in *Potentilla*. *Hereditas* 102, 289–292. doi: 10.1111/j.1601-5223.1985.tb00628.x

Beaulieu, J. M., and O'Meara, B. C. (2016). Detecting Hidden Diversification Shifts in Models of Trait-Dependent Speciation and Extinction. *Syst. Biol.* 65, 583–601. doi: 10.1093/sysbio/syw022

Beaulieu, J. M., O'Meara, B. C., and Donoghue, M. J. (2013). Identifying Hidden Rate Changes in the Evolution of a Binary Morphological Character: The Evolution of Plant Habit in Campanulid Angiosperms. *Syst. Biol.* 62, 725–737. doi: 10.1093/sysbio/syt034

Briscoe, A. D., and Chittka, L. (2001). The Evolution of Color Vision in Insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Brock, M. T., Lucas, L. K., Anderson, N. A., Rubin, M. J., Markelz, R. J. C., Covington, M. F., et al. (2016). Genetic architecture, biochemical underpinnings and ecological impact of floral UV patterning. *Mol. Ecol.* 25, 1122–1140. doi: 10.1111/mec.13542

Bull, J. J., and Charnov, E. L. (1985). On Irreversible Evolution. *Evolution* 39, 1149–1155. doi: 10.1111/j.1558-5646.1985.tb00455.x

Conner, J. K., and Rush, S. (1996). Effects of flower size and number on pollinator visitation to wild radish, *Raphanus raphanistrum*. *Oecologia* 105, 509–516. doi: 10.1007/BF00330014

de Jager, M. L., Willis-Jones, E., Critchley, S., and Glover, B. J. (2017). The impact of floral spot and ring markings on pollinator foraging dynamics. *Evol. Ecol.* 31, 193–204. doi: 10.1007/s10682-016-9852-5

Ding, B., Patterson, E. L., Holalu, S.V., Li, J., Johnson, G. A., Stanley, L. E., et al. (2020). Two myb proteins in a self-organizing activator-inhibitor system produce spotted pigmentation patterns. *Curr. Biol.* 30 (5), 802–814.

Dinkel, T., and Lunau, K. (2001). How drone flies (*Eristalis tenax* L., Syrphidae, Diptera) use floral guides to locate food sources. *J. Insect Physiol.* 47, 1111–1118. doi: 10.1016/S0022-1910(01)00080-4

Dyer, A. G. (1996). Reflection of Near-Ultraviolet Radiation From Flowers of Australian Native Plants. *Aust. J. Bot.* 44, 473–488. doi: 10.1071/bt9960473

Free, J. B. (1970). Effect of Flower Shapes and Nectar Guides on the Behaviour of Foraging Honeybees. *Behaviour* 37, 269–285. doi: 10.1163/156853970X00376

Goodwin, T. (1980). *The Biochemistry of the Carotenoids: Volume I Plants* (Springer Science and Business Media).

Gould, S. J. (1970). Dollo on Dollo's law: Irreversibility and the status of evolutionary laws. *J. Hist. Biol.* 3 (2), 189–212. doi: 10.1007/BF00137351

Gronquist, M., Bezzerides, A., Attygalle, A., Meinwald, J., Eisner, M., and Eisner, T. (2001). Attractive and defensive functions of the ultraviolet pigments of a flower (*Hypericum calycinum*). *Proc. Natl. Acad. Sci.* 98, 13745–13750. doi: 10.1073/pnas.231471698

Guldberg, L. D., and Atsatt, P. R. (1975). Frequency of Reflection and Absorption of Ultraviolet Light in Flowering Plants. *Am. Midland Nat.* 93, 35–43. doi: 10.2307/2424103

Hansen, D. M., Van der Niet, T., and Johnson, S. D. (2012). Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proc. R. Soc. B.: Biol. Sci.* 279, 634–639. doi: 10.1098/rspb.2011.1349

Harborne, J. B., and Nash, R. J. (1984). Flavonoid pigments responsible for ultraviolet patterning in petals of the genus *Potentilla*. *Biochem. Syst. Ecol.* 12, 315–318. doi: 10.1016/0305-1978(84)90055-3

Hempel de Ibarra, N., and Vorobyev, M. (2009). Flower patterns are adapted for detection by bees. *J. Comp. Physiol. A* 195, 319–323. doi: 10.1007/s00359-009-0412-0

Hempel de Ibarra, N., Langridge, K. V., and Vorobyev, M. (2015). More than colour attraction: behavioural functions of flower patterns. *Curr. Opin. Insect Sci.* 12, 64–70. doi: 10.1016/j.cois.2015.09.005

Hirota, S. K., Miki, N., Yasumoto, A. A., and Yahara, T. (2019). UV bull's-eye contrast of *Hemerocallis* flowers attracts hawkmoths but not swallowtail butterflies. *Ecol. Evol.* 9, 52–64. doi: 10.1002/ece3.4604

Jiang, P., and Rausher, M. (2018). Two genetic changes in cis-regulatory elements caused evolution of petal spot position in *Clarkia*. *Nat. Plants* 4, 14–22. doi: 10.1038/s41477-017-0085-6

Johnson, S. D., and Dafni, A. (1998). Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Funct. Ecol.* 12, 289–297. doi: 10.1046/j.1365-2435.1998.00175.x

Johnson, S. G., Delph, L. F., and Elderkin, C. L. (1995). The effect of petal-size manipulation on pollen removal, seed set, and insect-visitor behavior in *Campanula americana*. *Oecologia* 102, 174–179. doi: 10.1007/BF00333249

Jones, C. E., Dorsett, D. K., Roelofs, F. M., and Shah, C. V. (1999). Ultraviolet Floral Patterns in the Native Hawaiian Flora: What Do They Mean for Island Biogeography? *Pac. Sci.* 53, 82–87.

Jorgensen, R. A. (1995). Cosuppression, Flower Color Patterns, and Metastable Gene Expression States. *Science* 268, 686–691. doi: 10.1126/science.268.5211.686

Kelber, A. (1997). Innate preferences for flower features in the hawkmoth. *J. Exp. Biol.* 200, 827–836.

Koski, M. H., and Ashman, T.-L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct. Ecol.* 28, 868–877. doi: 10.1111/1365-2435.12242

Koski, M. H., and Ashman, T.-L. (2015b). An altitudinal cline in UV floral pattern corresponds with a behavioral change of a generalist pollinator assemblage. *Ecology* 96, 3343–3353. doi: 10.1890/15-0242.1

Koski, M. H., and Ashman, T.-L. (2015a). Floral pigmentation patterns provide an example of Gloger's rule in plants. *Nat. Plants* 1, 1–5. doi: 10.1038/nplants.2014.7

Koski, M. H., and Ashman, T.-L. (2016). Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors. *New Phytol.* 211, 708–718. doi: 10.1111/nph.13921

Lawson, D. A., and Rands, S. A. (2018). The evolution of floral guides: using a genetic algorithm to investigate the evolution of floral cue arrangements. *Biol. J. Linn. Soc.* 123, 739–753. doi: 10.1093/biolinnean/bly011

Lehrer, M., Horridge, G. A., Zhang, S. W., and Gadagkar, R. (1995). Shape vision in bees: innate preference for flower-like patterns. *Philos. Trans. R. Soc. London Ser. B.: Biol. Sci.* 347, 123–137. doi: 10.1098/rstb.1995.0017

Leonard, A. S., and Papaj, D. R. (2011). 'X' marks the spot: The possible benefits of nectar guides to bees and plants. *Funct. Ecol.* 25, 1293–1301. doi: 10.1111/j.1365-2435.2011.01885.x

Leonard, A. S., Brent, J., Papaj, D. R., and Dornhaus, A. (2013). Floral Nectar Guide Patterns Discourage Nectar Robbing by Bumble Bees. *PloS One* 8. doi: 10.1371/journal.pone.0055914

Lunau, K., Fieselmann, G., Heuschen, B., and van de Loo, A. (2006). Visual targeting of components of floral colour patterns in flower-naïve bumblebees (*Bombus terrestris*; Apidae). *Naturwissenschaften* 93, 325–328. doi: 10.1007/s00114-006-0105-2

Lunau, K., Unseld, K., and Wolter, F. (2009). Visual detection of diminutive floral guides in the bumblebee *Bombus terrestris* and in the honeybee *Apis mellifera*. *J. Comp. Physiol. A* 195, 1121–1130. doi: 10.1007/s00359-009-0484-x

Lunau, K. (2006). Stamens and mimic stamens as components of floral colour patterns. *Botanische Jahrbücher* 127, 13–41. doi: 10.1127/0006-8152/2006/0127-0013

Manning, A. (1956). The Effect of Honey-Guides. *Behaviour* 9, 114–139. doi: 10.1163/156853956X00273

McCrea, K. D., and Levy, M. (1983). Photographic Visualization of Floral Colors as Perceived by Honeybee Pollinators. *Am. J. Bot.* 70, 369–375. doi: 10.1002/j.1537-2197.1983.tb06404.x

Mol, J., Grotewold, E., and Koes, R. (1998). How genes paint flowers and seeds. *Trends Plant Sci.* 3, 212–217. doi: 10.1016/S1360-1385(98)01242-4

Ng, J., and Smith, S. D. (2014). How traits shape trees: new approaches for detecting character state-dependent lineage diversification. *J. Evol. Biol.* 27 (10), 2035–2045.

Pagel, M. (1994). Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proc. R. Soc. London Ser. B.: Biol. Sci.* 255, 37–45. doi: 10.1098/rspb.1994.0006

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A. R., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18 (1), 46–55. doi: 10.1111/plb.12322

Paradis, E., and Claude, J. (2002). Analysis of Comparative Data Using Generalized Estimating Equations. *J. Theor. Biol.* 218, 175–185. doi: 10.1006/jtbi.2002.3066

Penny, J. H. J. (1983). Nectar Guide Colour Contrast: A Possible Relationship with Pollination Strategy. *New Phytol.* 95, 707–721. doi: 10.1111/j.1469-8137.1983.tb03534.x

Peterson, M. L., Miller, T. J., and Kay, K. M. (2015). An ultraviolet floral polymorphism associated with life history drives pollinator discrimination in *Mimulus guttatus*. *Am. J. Bot.* 102, 396–406. doi: 10.3732/ajb.1400415

Quattrocchio, F., Baudry, A., Lepiniec, L., and Grotewold, E. (2006). "The Regulation of Flavonoid Biosynthesis," in *The Science of Flavonoids*. Ed. E. Grotewold (New York, NY: Springer), 97–122. doi: 10.1007/978-0-387-28822-2_4

Ree, R. H., and Donoghue, M. J. (1999). Inferring Rates of Change in Flower Symmetry in Asterid Angiosperms. *Syst. Biol.* 48, 633–641. doi: 10.1080/106351599260201

Rivkin, L. R., Case, A. L., and Caruso, C. M. (2016). Why is gynodioecy a rare but widely distributed sexual system? Lessons from the Lamiaceae. *New Phytol.* 211, 688–696. doi: 10.1111/nph.13926

Smith, S. D., and Goldberg, E. E. (2015). Tempo and mode of flower color evolution. *Am. J. Bot.* 102, 1014–1025. doi: 10.3732/ajb.1500163

Smith, S. D. (2010). Using phylogenetics to detect pollinator-mediated floral evolution. *New Phytol.* 354–363. doi: 10.1111/j.1469-8137.2010.03292.x

Stern, D. L., and Orgogozo, V. (2008). The loci of evolution: how predictable is genetic evolution? *Evolution* 62, 2155–2177. doi: 10.1111/j.1558-5646.2008.00450.x

Stevens, P. F., and Smith, S. D. (2001). Angiosperm Phylogeny Website. Version 14, July 2017. Availale at:http://www.mobot.org/MOBOT/research/APweb/

Verhoeven, C., Ren, Z.-X., and Lunau, K. (2018). False-colour photography: a novel digital approach to visualize the bee view of flowers. *J. Pol. Ecol.* 23, 102–118. doi: 10.26786/1920-7603(2018)11

Waser, N. M., and Price, M. V. (1985). The effect of nectar guides on pollinator preference: experimental studies with a montane herb. *Oecologia* 67, 121–126. doi: 10.1007/BF00378462

Wessinger, C. A., Rausher, M. D., and Hileman, L. C. (2019). Adaptation to hummingbird pollination is associated with reduced diversification in Penstemon. *Evol. Lett.* 3, 521–533. doi: 10.1002/evl3.130

Zufall, R. A., and Rausher, M. D. (2004). Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* 428, 847–850. doi: 10.1038/nature02489

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Geographic Variation in Floral Color and Reflectance Correlates With Temperature and Colonization History

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Petal color variation within species is common and may be molded by abiotic or biotic selection pressures, or neutral population structure. For example, darker flowers may be favored in cooler environments because they absorb more solar radiation, elevating the temperature of reproductive structures. Additionally, flower color may evolve to attract the dominant or most efficient pollinator type in a given population. Here, we evaluate geographic variation in petal coloration across the range of *Campanula americana* in Eastern North America and test whether color covaries with abiotic factors, the pollination community, and genetic structure established through post-glacial expansion. Consistent with other studies, flowers from cooler, higher latitude populations were less reflective across the UV-NIR spectrum than those from warmer populations. Local temperature explained variation in petal reflectance better than the pollinator community or colonization history. Petal color perceived by trichromatic bee pollinators displayed a strong longitudinal pattern but was unassociated with climatic factors and the pollinator community. Instead, pollinator-perceived color was tightly correlated with the geographic distance from *C. americana*'s glacial refugium. In total, abiotic conditions appear to shape large-scale geographic variation in the intensity of petal reflectance while genetic structure is the strongest driver of pollinator-perceived petal coloration. This study highlights the importance of abiotic factors and historical processes associated with range expansion as major evolutionary forces shaping diversity of flower coloration on large geographic scales.

Keywords: biogeography, genetic drift, flower color, insect vision, range expansion, thermoregulation

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INTRODUCTION

Floral coloration can have strong effects on plant-pollinator interactions (e.g., Gigord et al., 2001; Guitián et al., 2017), and recent evidence highlights its impact on the microclimate experienced by pollen and ovules (Lacey et al., 2010; Koski and Ashman, 2015; van der Kooi et al., 2019). Correlating geographic variation in petal coloration with ecological parameters such as pollinator communities or climatic conditions is one approach that can elucidate the degree to which putative

selective agents may shape the evolution of color across large spatial scales (Brunet, 2009; Sobral et al., 2015). However, neutral population genetic structure has the potential to contribute to geographic variation in petal color as well (Rausher, 2008). For instance, genetic structuring due to range expansion and contraction have been posited to shape geographic petal color variation in *Gentiana lutea* (Sobral et al., 2015). An understanding of both adaptive and non-adaptive processes can be crucial for explaining floral color evolution (Streisfeld and Kohn, 2005; Streisfeld and Kohn, 2007; Hopkins et al., 2012; Baranzelli et al., 2014; Berardi et al., 2016).

Pollinators select on flower color attributes such as brightness and hue (Caruso et al., 2010; Sletvold et al., 2016), so the most commonly tested driver of geographic variation in floral coloration is the pollination community. Pollinator guilds often differ in their sensory biases for flower color (Schiestl and Johnson, 2013) and if pollinator communities differ between populations they can drive geographically divergent selection. There is strong evidence that pollinators contribute to flower color disparities among populations of the same species in some systems (Streisfeld and Kohn, 2007; Sobral et al., 2015; Streinzer et al., 2019). On the other hand, differences in pollinator communities are insufficient for explaining color variation in a number of others (Schemske and Bierzychudek, 2007; Thairu and Brunet, 2015). Thus, non-pollinator agents of selection are often invoked to shape variation in flower coloration (e.g., Strauss and Whittall, 2006).

Abiotic conditions such as temperature (Coberly and Rausher, 2003; Lacey et al., 2010; Koski and Galloway, 2018), water availability (Warren and Mackenzie, 2001; Arista et al., 2013; Vaidya et al., 2018), or solar radiation (Arista et al., 2013; Koski and Ashman, 2015), can act as selective agents on flower color either directly or indirectly. As a result, large scale patterns of flower color have been linked with climatic gradients (Arista et al., 2013; Koski and Ashman, 2015; Koski and Galloway, 2018). For example, less reflective (darker) flowers have the ability to warm reproductive structures more efficiently than lighter flowers through absorption of more solar radiation, which can increase reproductive success in cooler climates (Lacey et al., 2010). Pollinators have also been shown to prefer warmer flowers in cooler environments (van der Kooi et al., 2019), potentially providing an advantage to darker flowers. Darker coloration can also be favored in drought conditions (Warren and Mackenzie, 2001). For example, in *Boechera stricta*, there is a greater likelihood of pigmented flowers in low elevation populations which may be due to elevated drought tolerance of pigmented morphs (Vaidya et al., 2018). Models that incorporate the effects of both abiotic and pollinator attributes of the environment on coloration can help to parse the impacts of each on floral color.

It is also important to consider the effects of population genetic structure when examining large-scale geographic covariance between coloration and ecological factors (Rausher, 2008). Numerous studies have found natural selection is stronger than drift in driving among-population floral color variation (Schemske and Bierzychudek, 2007; Streisfeld and Kohn, 2007; Jorgensen et al., 2006; Hopkins et al., 2012). However, to date very few studies have examined the effects of population structure imposed by historical colonization on petal color variation across the range of a species (however see Baranzelli et al., 2014). During range expansion, small founder populations that establish beyond a range edge are most likely derived from range-edge populations (Hallatschek and Nelson, 2008). Serial genetic bottlenecks experienced through founder events should establish geographic population structure with genetic diversity declining with increasing distance from glacial refugia (e.g., Willi et al., 2018; Koski et al., 2019). Floral color could show spatial patterns consistent with neutral evolution along expansion routes. In this scenario, geospatial variation in color would be a signature of historical processes rather than geographically divergent natural selection, though both scenarios are not mutually exclusive.

Campanula americana is a widespread plant in Eastern North America with petals that range from blue to violet. It is pollinated by bumblebees which are very effective at pollen transfer, and small solitary bees that are relatively ineffective pollinators (Koski et al., 2018). It displays variation in pollen coloration that is genetically uncorrelated with petal color (Koski and Galloway, 2018; Koski et al., 2020). Geographic patterns of petal coloration have not been evaluated. Here, we correlate range-wide variation in signatures of petal reflectance with data on pollinator communities, climatic variables, and post-glacial expansion for 24 populations of *C. americana*. We address the following questions: 1) Does petal coloration display geographic variation? and 2) If so, is it driven by pollinator communities, climatic variables, or historical range expansion? If selection is important in shaping color variation, we predict that a) petals will be less reflective in more northern populations where elevated absorbance is likely favored in cool environments, and b) petal color as perceived by pollinators will be correlated with the relative abundances of effective bumblebee pollinators and ineffective small solitary bees. Alternatively, if genetic structure imposed by post-glacial colonization is more important in structuring color variation than contemporary selection, we predict that the distance from the glacial refugium will strongly predict all aspects of petal coloration.

METHODS

System

Campanula americana (=*Campanulastrum americanum*, Campanulaceae) is a bee-pollinated annual to biennial species of Eastern North America that flowers from late June to early September. It occurs frequently at forest edges and less commonly in the understory and spans a large environmental gradient from Florida to Minnesota. It is largely outcrossing (Galloway et al., 2003; Koski et al., 2019) and is visited by a variety of bee species with varying pollination effectiveness. Bumblebees are highly effective at pollen transfer and affect higher seed set per flower visit than smaller solitary bees (*Megachile campanulae* (Megachilidae); various Halictidae; Lau and Galloway, 2004; Koski et al., 2018). Across its range west of

the Appalachian Mountains, it is pollen limited on average (mean for 24 populations= 19%; Koski et al., 2017). However, populations that experience higher bumblebee visitation rates are less pollen limited (Koski et al., 2017; Koski et al., 2018). The color of pollen varies from white to deep purple (Lau and Galloway, 2004) and darker pollen has elevated germination potential under heat stress (Koski and Galloway, 2018). Bumblebees learn to associate rewards with pollen color, and in natural populations, *M. campanulae* prefers flowers with darker pollen (Ison et al., 2019). Thus, color variation has the ability to impact pollinator choice. Pollen color and petal color are not genetically correlated (Koski et al., 2020).

In this study we focus on 24 populations west of the Appalachian Mountains which form a clade that is divergent from populations in the Appalachians, as well as those east of the Appalachians (Barnard-Kubow et al., 2015). Population genomic analyses support an origin for this Western clade in the Appalachian Plateau of Southwestern Kentucky (Koski et al., 2019). This location served as the most-likely region of glacial refugia during the Pleistocene glaciation with stepwise colonization westward and northward following glacial recession. Further genomic analyses support genetic clustering west and east of the Mississippi River (Prior et al., in revision).

Plant Material and Petal Reflectance Measurements

Ripe fruits were field collected from the 24 C. *americana* populations in Summer 2015. We sowed seed from 25 maternal families from each population in growth chambers at the University of Virginia. A single seedling from each family was vernalized and then transplanted into the greenhouse (see Koski et al., 2017 for detail on plant propagation). Upon flowering, we collected a single first day flower from an average of 15.2 plants per population (n=365 plants). Using an Ocean Optics spectrometer with a UV-Vis light source, we measured petal reflectance with a reflection probe held at a 90 angle. Since petals are not glossy, specular reflection was not likely to contribute strongly to spectral outputs. Within a given plant, flowers are similar in coloration (M. Koski, pers. obs.).

Campanula americana petals display a peak reflectance in the blue range around 430nm, reduced reflectance between 500 and 650nm and an increase in reflectance in the NIR range (**Figure 1**). UV reflectance from petals is low in these populations west of the Appalachians. We first scored reflectance metrics that captured variation in reflectance curves but did not take into consideration pollinator visual systems. These included Mean Reflectance (i.e., mean brightness), Peak Blue Reflectance, and Peak NIR reflectance. Mean reflectance is the average % reflectance from 300-850 nm. Peak blue reflectance is the highest % reflectance value recorded between 410 and 500 nm, and NIR reflectance is the % reflectance recorded at 800 nm. Similar metrics have been used as estimates for the potential for heat absorption in other systems (e.g., Lacey and Herr, 2005). We chose to measure NIR reflectance because long wavelengths have the potential to contribute to heat gain despite often being ignored because they do not contribute to the visual perception of color (Stuart-Fox et al., 2017). Measurements of reflectance were averaged for each population.

We then modeled petal color from each plant in hexagonal color space using peak photoreceptor absorbance for *Bombus impatiens,* a known pollinator of *C. americana*. Peak absorbances for this trichromatic visual system are 347, 424, and 539nm (Skorupski and Chittka, 2010). Photoreceptor absorbance is not known for another common pollinator of *C. americana*, *Megachile campanulae* (Megachilidae). We therefore used visual system data from a confamilial taxon, *Osmia rufa* (348,

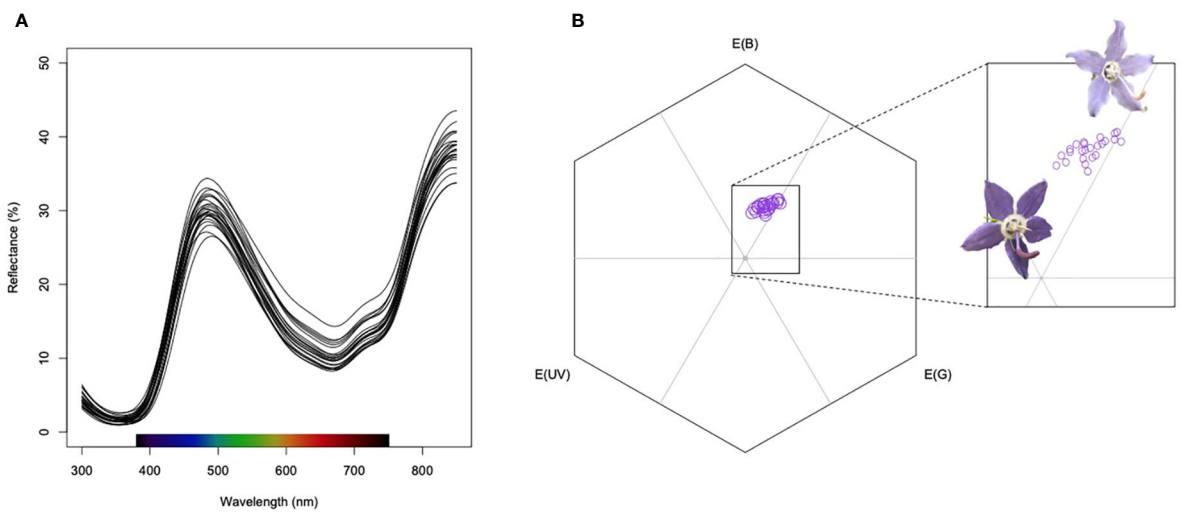


FIGURE 1 | (A) Spectral reflectance of petals of *Campanula americana*. Each curve is the average spectral reflectance for a single population (N=24). **(B)** Mean spectral reflectance for each population placed in hexagonal color space of the trichromatic pollinator *Bombus impatiens*. The inset focuses on the blue and blue-green sectors of color space in which *C. americana* petals fall. Flower images display petals with a low *x* and low *y* coordinate (deeper purple) and petals with higher *x* and *y* coordinates (lighter purple).

436, and 572 nm; Menzel et al., 1988) to approximate petal color perceived by *M. campanulae*. For each pollinator visual system, we modeled petal color using the long-wavelength photoreceptor for achromatic receptor stimulation, standard D65 illuminance, a standard green background, hyperbolic-transformed quantum catch, and a von Kries color correction using the Pavo package in R (Maia et al., 2019). Each visual model was plotted into hexagonal color space to obtain *x* and *y* coordinates. The coordinates in color space were averaged across individuals for each population.

Predictors of Color Variation: Climatic Data, Pollinator Data, and Population Structure

We extracted average temperature (bio10) and precipitation (bio18) of the warmest quarter from Worldclim (Hijmans et al., 2005) using the latitude and longitude of each population. We chose average summer climatic values because they are representative of the conditions experienced by flowers (see Koski and Galloway, 2018). In particular, temperature and drought are two factors shown to impose selection on anthocyanin-based petal coloration (e.g., Warren and Mackenzie, 2001; Lacey and Herr, 2005; Vaidya et al., 2018). Although other work has found duration of solar radiation influences floral color (Arista et al., 2013), preliminary analysis found no relationship for *C. americana* (results not shown).

We observed pollinator visitation to flowers in 1 m^2 plots in each of the 24 focal populations in 2015 and 2016. In each year we observed six plots per population for 15 min each and counted the number of open flowers. We categorized visitors as small bees or large bees. The small bee category includes the common visitor *M. campanulae* and other solitary bees (see Koski et al., 2017 for detail). The bees in this category are active pollen collectors and are relatively inefficient pollinators relative to *Bombus* spp. (Koski et al., 2018). The large bee category consists almost exclusively of *Bombus* spp. Average visitation rate for each population across two years for each category was calculated as visits per flower per hour. One population was inaccessible in 2016, so only had one year of pollinator visitation data (Arkansas 56). Visitation rate did not differ between years (Koski et al., 2017).

Previous phylogenomic work based on RAD-Sequencing of 24 populations found populations of *C. americana* west of the Appalachians (i.e., those in the current study) originated from a glacial refugium in Southeastern Kentucky. The geographic distance from this refugium predicts population-level genetic diversity and both genomic and ecological genetic estimates of genetic drift (Koski et al., 2019), strongly supporting post-glacial expansion from this area. Thus, populations are genetically-structured with increasing distance from the refugium (Koski et al., 2019; Prior et al. in review). We used the linear geographic distance from the refugium as a proxy of population structure established through post-glacial migration (Koski et al., 2019).

Statistical Analyses

To determine whether mean petal reflectance, blue reflectance, and NIR reflectance displayed clinal geographic variation across the range of *C. americana*, we modeled the population average of each metric as a function of latitude and longitude with multiple linear regressions. We then asked whether climatic, pollinator, or genetic structure contributed to any geographic variation. We modeled each reflectance parameter as a function of two climatic variables (summer temperature, summer precipitation), small bee visitation rate, *Bombus* visitation rate, and the distance from the glacial refugium using multiple linear regression. Pollinator visitation rates were log + 0.1 transformed to improve normality.

For petal color modeled in hexagonal pollinator visual space, the average population-level x- and y-coordinates were correlated (*Bombus* model, r=0.64, $P < 0.001$; *Osmia* model, r=0.66, $P < 0.001$). Therefore, we modeled the location in hexagonal visual space (*x* and *y*) using MANOVA. All MANOVA models were the same as those used to model petal reflectance measures. All statistical analyses were performed in R (R Core Team, 2020).

RESULTS

Spatial Variation in Petal Reflectance and Color

Together, latitude and longitude explained 24% of the variation in mean petal reflectance across 24 populations of *C. americana* ($F_{2,21} = 3.35$, P=0.054, $R^2 = 0.24$), but only the effect of latitude was significant (**Table 1**). Petals in more northerly populations displayed lower average reflectance from 300 to 850 nm (**Figure 2**). That is, more northern populations had darker petals. Similar patterns were observed for peak blue and NIR reflectance (**Table 1**).

Petal coloration in hexagonal visual space for *Bombus impatiens* displayed a strong longitudinal cline (**Table 1**). Populations with higher x-axis values tended to be near or within the blue-green sector of hexagonal visual space, while those with lower values were in the center of the blue sector (**Figure 1**). Thus, we visualized variation in the x-coordinate across the range (**Figure 3**). The x-coordinate in visual space decreased from west to east across the range (**Figure 3**). Results obtained using *Osmia rufa*'s visual system were similar (**Supplementary Table 1**). Thus, overall petal reflectance covaried with latitude, while petal color modeled using pollinator visual systems covaried with longitude.

Ecological and Historical Predictors of Reflectance and Color Variation

In the model testing the effects of climatic variables, the pollinator community, and the distance from the glacial refugium on petal coloration, only temperature predicted petal reflectance (**Table 2**; **Figure 4**). Temperature was positively associated with overall, blue, and NIR reflectance (**Table 2**).

TABLE 1 | Spatial variation in petal reflectance attributes and pollinator-perceived color for 24 populations of *Campanula americana*.

	Mean Reflectance			Blue Reflectance			NIR Reflectance			Location in Hexagonal Color Space *Bombus impatiens*		
	Estimate	**t**	***P***	**Estimate**	**t**	***P***	**Estimate**	**t**	***P***	**Pillai's Trace**	**$F_{2,20}$**	***P***
Intercept	27.251	4.024	0.001	34.084	3.987	0.001	35.810	3.194	0.004	–	–	–
Latitude	**-0.213**	**-2.514**	**0.020**	**-0.245**	**-2.295**	**0.032**	**-0.312**	**-2.225**	**0.037**	0.169	2.030	0.157
Longitude	-0.050	-0.736	0.470	-0.065	-0.758	0.457	-0.185	-1.637	0.116	**0.531**	**11.330**	**<0.001**
	R^2 = 0.24; $F_{2,21}$ = 3.35; P=0.054			R^2 = 0.21; $F_{2,21}$ = 2.843; P=0.08			R^2 = 0.26; $F_{2,21}$ = 3.65; P=0.04					

Reflectance was analyzed using multiple linear regression while the x-y coordinates of hexagonal color space for Bombus impatiens were modeled using MANOVA with Type II sum of squares.
Statistics in bold font are significant at the P < 0.05 level.

When petal reflectance was modeled with trichromatic pollinator visual systems, there was a strong effect of the distance from the glacial refugium on the location of petal color in pollinator visual space (**Table 2**). With increasing distance from the refugium, populations had smaller x- and y-coordinates in hexagonal color space of *Bombus impatiens* (**Figure 5**). Additionally, there was a marginally significant effect of small bee visitation on pollinator-perceived color (p=0.06; **Table 2**). Specifically, flowers had smaller x and y coordinates in populations with higher small bee visitation (**Supplementary Figure 1**). Results were consistent when the visual system of *Osmia rufa* was used to model color (**Supplementary Table 2**).

DISCUSSION

Range-wide geographic patterns of petal coloration in *C. americana* appear to be largely shaped by the thermal environment and genetic structuring imposed by post-glacial range expansion. Petal reflectance, which has been shown to function in floral thermoregulation in other systems, displayed a

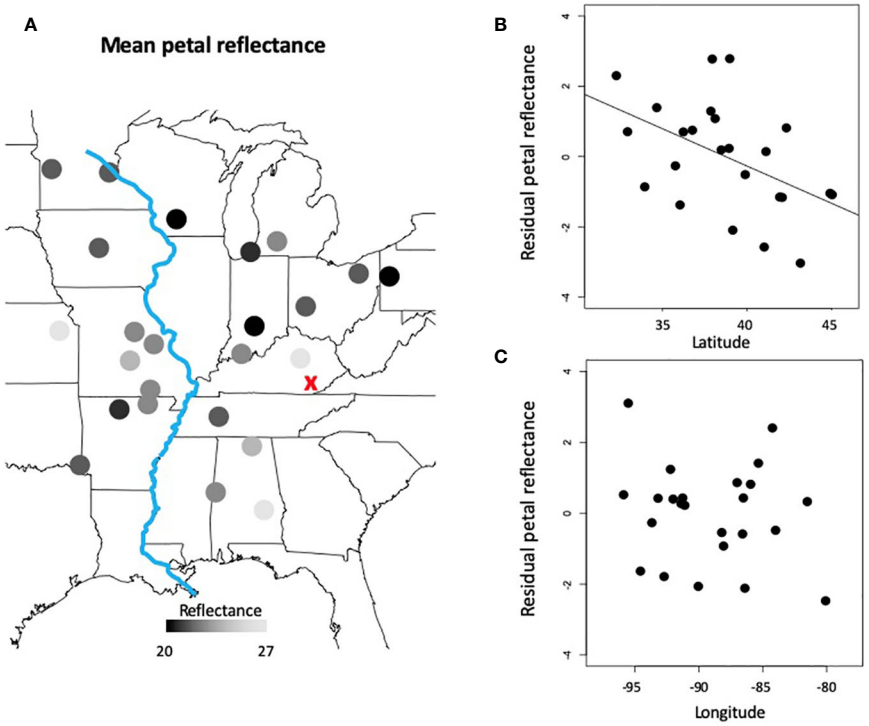


FIGURE 2 | Spatial variation in mean petal reflectance among populations. **(A)** A heat map of petal reflectance across *Campanula americana*'s range with darker circles indicating less reflective petals. The red 'X' is the most likely region of the Pleistocene glacial refugium of *C. americana*, and the blue line is the Mississippi River. **(B)** The direct effect of latitude on petal reflectance after accounting for longitudinal variation. **(C)** The direct effect of longitude on petal reflectance after accounting for latitudinal variation.

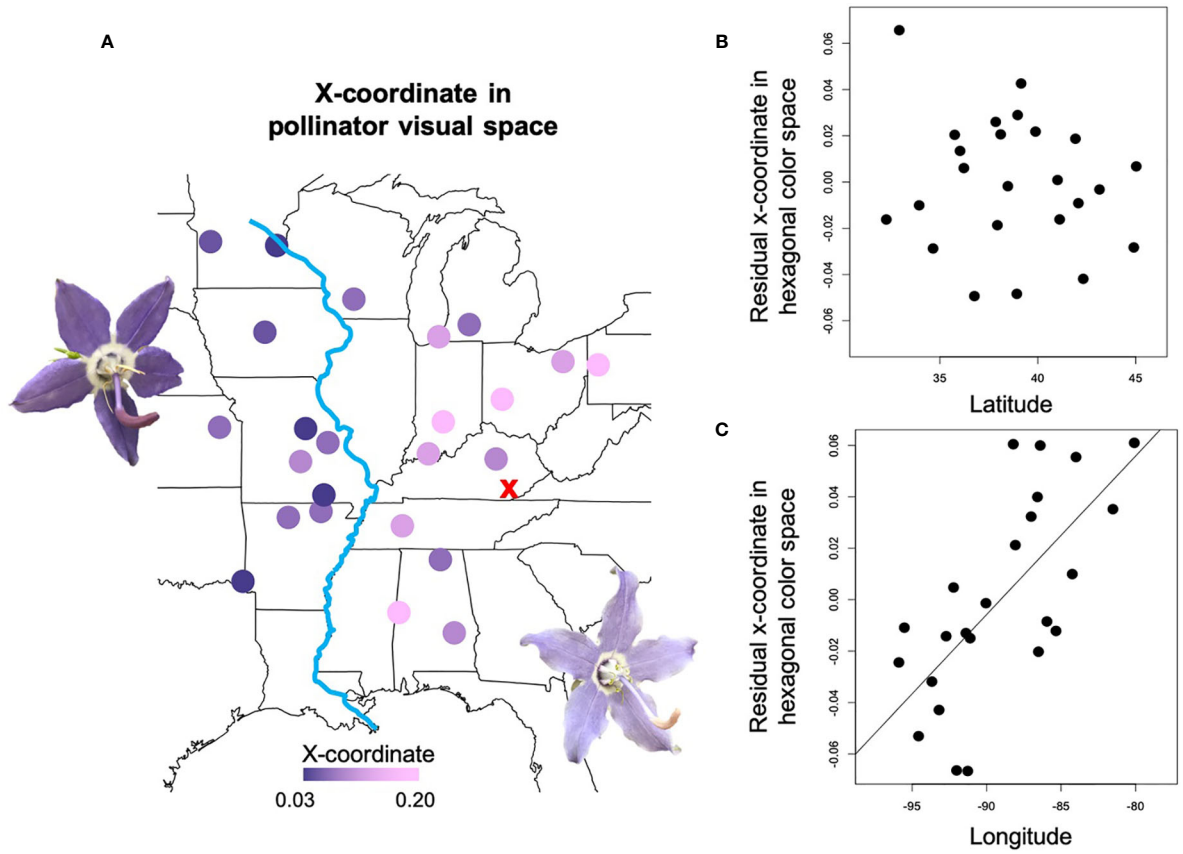


FIGURE 3 | Spatial variation in petal color perceived by trichromatic *Bombus impatiens*. **(A)** A heat map of the x-coordinate in hexagonal color space for *Campanula americana*'s range with darker circles indicating lower x values. X-coordinate values are shown on the map because they are more variable among populations than y-coordinates. The red "X" is the most likely region of the Pleistocene glacial refugium of *C. americana*, and the blue line is the Mississippi River. Colors roughly represent the human-perceived color of petals. **(B)** The direct effect of latitude on the x-coordinate after accounting for longitudinal variation. **(C)** The direct effect of longitude on the x-coordinate after accounting for latitudinal variation.

TABLE 2 | Effects of temperature, precipitation, pollinator visitation, and post-glacial colonization on petal reflectance attributes and pollinator-perceived color for 24 populations of *Campanula americana*.

	Mean Reflectance			**Blue Reflectance**			**NIR Reflectance**			**Location in Hexagonal Color Space, *Bombus impatiens***		
	Estimate	**t**	***P***	**Estimate**	**t**	***P***	**Estimate**	**t**	***P***	**Pillai's Trace**	**$F_{2,17}$**	***P***
Intercept	8.685	1.568	0.134	12.625	1.802	0.088	18.065	1.920	0.071	–	–	–
Summer Temp.	**0.452**	**2.854**	**0.011**	**0.519**	**2.595**	**0.018**	**0.757**	**2.814**	**0.012**	0.135	1.322	0.293
Summer Precipitation	0.014	1.037	0.313	0.018	1.109	0.282	0.007	0.328	0.747	0.166	1.693	0.214
Small bee visitation	0.260	0.697	0.495	0.269	0.570	0.576	0.820	1.296	0.211	0.276	3.246	0.064
***Bombus* visitation**	-0.145	-0.528	0.604	-0.068	-0.197	0.846	-0.248	-0.533	0.601	0.078	0.718	0.502
Km from Refugium	0.000	-0.459	0.652	0.000	-0.360	0.723	0.001	0.505	0.620	**0.603**	**12.936**	**<0.0001**
	$R^2 = 0.38$; $F_{5,18} = 2.17$; $P=0.104$			$R^2 = 0.35$; $F_{5,18} = 1.94$; $P=.138$			$R^2 = 0.36$; $F_{5,18} = 1.99$; $P=0.128$					

Reflectance was analyzed using multiple linear regression while the x-y coordinates of hexagonal color space for Bombus impatiens were modeled using MANOVA with Type II sum of squares.
Bold values indicate significance at $P < 0.05$.

latitudinal pattern with petals being less reflective in colder environments. However, petal color modeled with trichromatic pollinator visual systems displayed a strong longitudinal pattern that is largely consistent with flower color evolving in concert with post-glacial range expansion. The role of more contemporary pollinator environments in driving color variation is less important than the role of historical expansion, a finding that is similar to the geographic structuring of other floral traits in *C.*

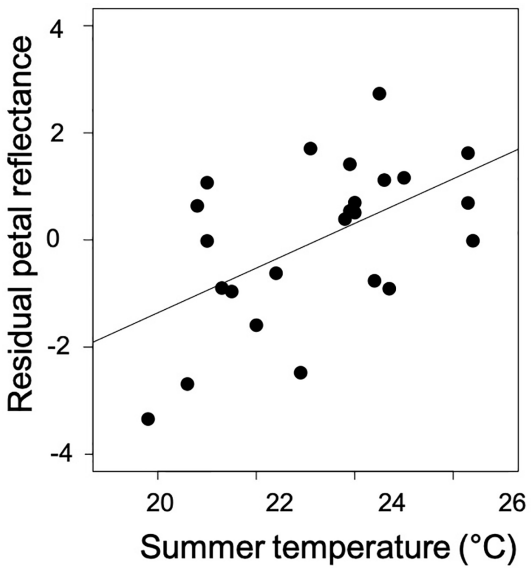


FIGURE 4 | The direct effect of average temperature of the warmest quarter on mean petal reflectance after accounting for precipitation, pollinator visitation, and the post-glacial colonization (distance from glacial refugium; see **Table 2**).

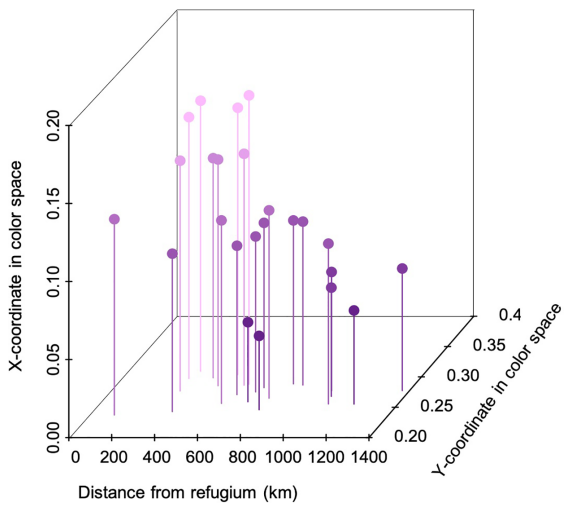


FIGURE 5 | The relationship between petal color perceived by trichromatic pollinator *Bombus impatiens,* and the distance from the glacial refugium. X and y- coordinates corresponds to **Figure 1B** and the location of the glacial refugium is denoted in **Figures 2** and **3**.

americana (e.g., selfing ability; Koski et al., 2017). Thus, this work underscores the importance of the combination of neutral and adaptive evolutionary forces when examining the evolution of flower color.

Temperature as a Driver of Latitudinal Variation in Petal Reflectance

As predicted, petals were less reflective across the UV to NIR spectrum in more northern populations that experience cooler conditions during flowering. The latitudinal pattern was only associated with temperature. That is, there was no detectable influence of the pollinator community, post-glacial expansion or precipitation on petal reflectance in our models. Interestingly, previous phylogenomic work in *C. americana* suggests the potential for distinct genetic lineages east and west of the Mississippi River, and if the dataset is split by this barrier petal reflectance in both groups is elevated in populations that experience higher temperatures (Eastern group, r=0.60, *P*=0.037; Western group, r=0.53, *P*=0.078). These correlations bolster support for temperature contributing to the evolution of petal reflectance in *C. americana*. In *Plantago lanceolata*, cold temperatures result in increased floral anthocyanin production in inflorescences, increasing absorption, especially in the NIR range (Stiles et al., 2007). Increased light absorption warms reproductive structures while increased reflectance can cool reproductive structures or simply minimize solar heat gain (Lacey and Herr, 2005). Our results join Lacey et al. (2010) in showing a strong latitudinal pattern of reduced reflectance in cooler, northern populations. Since *C. americana* were grown in a common environment, the reflectance measurements capture fixed genetic differences among populations.

The pattern of reduced petal reflectance in cooler populations and elevated reflectance in warmer populations could be driven by a number of potential mechanisms. First, floral warming and cooling could benefit pollen and ovules in cold and warm conditions, respectively. Second, floral warming and cooling could increase pollinator visitation in cool and warm conditions, respectively. Finally, the latitudinal cline in petal reflectance could be due to plant-wide upregulation of the anthocyanin biosynthetic pathway under cooler temperatures, in which case there may be no adaptive role of variation in petal reflectance.

The viability of *C. americana* pollen is reduced by high heat stress, and lighter pollen color morphs (white/tan) incur more damage than darker purple pollen morphs (Koski and Galloway, 2018). Thus, increased petal reflectance, which has the potential to cool flowers (van der Kooi et al., 2019), should be favored in warmer southern populations that experience heat stress (Koski and Galloway, 2018). In contrast, an experimental cold temperature treatment (13°C) did not affect pollen viability in *C. americana,* therefore the effect of petal reflectance on warming in northern populations is unlikely driven by selection to increase pollen performance. Low temperatures have the ability to reduce ovule viability in other systems (e.g., Thakur et al., 2010). Thus, in cooler northern *C. americana* populations, increased absorption may warm flowers and increase ovule performance. However, given the inferior ovary of *C. americana* flowers, petal reflectance may have a minimal effect on ovule temperature.

Floral warming in the north and cooling in the south through altered petal reflectance could be the result of adaptation for increasing pollinator visitation. Warmer flowers experience elevated pollinator visitation in cold environments (Norgate et al., 2010). Conversely, pollinators have been shown to overheat in warmer flowers during extreme heat (Corbet and Huang, 2016), and choose cooler flowers at high temperatures (Shrestha et al., 2018). Small pollinators have been observed basking in flowers of *C. americana* (L. Galloway, pers. obs.), thus petal reflectance may affect pollinator thermal preferences with consequent impacts on plant reproductive success.

Finally, cold temperatures typically lead to upregulation of the anthocyanin biosynthetic pathway throughout the plant (Chalker-Scott, 1999) which could result in increased light absorption by petals in northern populations. Positive correlations in anthocyanin concentrations between petal and vegetative tissues can be strong (e.g., del Valle et al., 2015). In a mustard polymorphic for flower color, the frequency of the non-pigmented morph declined in the coldest populations, suggesting that anthocyanins are crucial for plant performance under cold stress (Dick et al., 2011). We posit that in *C. americana* darker petals have higher concentrations of anthocyanins and the latitudinal cline in petal reflectance could be driven by plant-wide adaptive responses to temperature across the wide latitudinal gradient.

Historical Colonization, Not Pollinators, as a Driver of Petal Color Variation

While mean petal reflectance displayed a latitudinal pattern, petal coloration modeled using the visual system of two separate trichromatic bee pollinators displayed a strong longitudinal pattern. The distance from the glacial refugium had the strongest effect on pollinator-perceived petal color, but there was also a modest and near-significant influence of small bee visitation rate. Historical migration of *C. americana* from the southern Appalachian plateau following glacial recession has resulted in genetic clusters of populations east and west of the Mississippi River (Koski et al., 2019; Prior et al. in revision). Geographic differences in flower color among populations are consistent with a Mississippi River split (**Figure 3**). Thus, we conclude that population structure driven by post-glacial expansion has been instrumental in shaping color variation in *C. americana*. To our knowledge, this is the first study to support that post-glacial migration contributes to geographic variation in a floral trait traditionally considered to influence pollinator attraction.

The link between petal color and post-glacial migration suggests that one of two evolutionary processes contributed to longitudinal petal color variation. First, neutral genetic drift occurred with range expansion, driving color evolution. Alternatively, novel selective pressures experienced in new habitats during colonization acted either directly on petal color or on traits correlated with petal color. Novel selection pressures may have included strong pollen and/or pollinator limitation or unique abiotic conditions. Alternatively, correlations between petal color and traits that are likely under strong selection during range expansion (like autonomous-selfing) may contribute to the structuring of petal color through range expansion. Among populations there is a correlation between the X-coordinate in *Bombus* color visual space and autonomous fruit set (r = -0.44, P= 0.03), though whether there is a functional link between selfing ability and coloration is unknown. For instance, the trait correlation could reflect independent evolution of each trait during range expansion, or a genetic correlation that did not evolve adaptively. The effects of neutral evolution during expansion and historical adaptation on the geographic pattern in flower color are difficult to disentangle. However, we predict that neutral genetic processes shaped the geographic pattern in petal color among *C. americana* populations, with any evolution due to contemporary pollinator or abiotic conditions occurring within that genetic structure, and hence resulting in a more modest impact on the phenotype.

We posit that alterations to petal color in *C. americana* are likely achieved through genetic modification to regulatory or structural anthocyanin biosynthetic pathway genes, resulting in a change in the ratios of colored anthocyanin compounds (i.e., copigmentation; Asen et al., 1972). While the pigment biochemistry of petals is not known, variation in pollen color in *C. americana* is driven largely by changes in concentrations of the anthocyanins cyanidin and peonidin (Koski et al., 2019). Populations east of the Mississippi River had petals closer to the "blue-green" sector of color space while those west of the Mississippi River had petals near the center of the "blue" sector of color space (**Figure 1**). This pattern was driven in part by a modest shift in the blue peak towards the pollinators mid-wavelength (green) photoreceptor in eastern populations. Populations with an average peak of reflectance near 440nm had higher x- and y- coordinates than populations with a peak near 435 nm (blue peak wavelength and x-coordinate correlation r=0.87, P < 0.0001; blue peak wavelength and y-coordinate correlation, r=0.41, P=0.05*).*Thus, the geographic pattern in pollinator-perceived color is due to a modest (5 nm) peak shift in reflectance in the blue range potentially driven by changes in copigmentation (Asen et al., 1972) that occurred in concert with range expansion.

Interestingly, pollen color in *C. americana* displays a longitudinal cline that has been largely attributed to selection by the thermal environment (Koski and Galloway, 2018). Specifically, pollen was deeper purple in western populations that experience higher summer temperatures and deeper purple pollen is more heat tolerant. However, Koski and Galloway (2018) scored pollen color by eye which does not incorporate reflectance of light outside of the wavelengths of human visual perception (~400-700 nm) nor does it model pollinator perception of color. Thus, the color metrics of petals from this study cannot be easily compared to the pollen color scored in Koski and Galloway (2018). Regardless, given the strong longitudinal cline in pollen color, there is the potential that historical colonization has played a role in structuring pollen color as well as petal color despite a lack of genetic correlation between the two (Koski et al., 2020).

CONCLUSIONS

Different components of petal reflectance appear to be shaped by different evolutionary forces in *C. americana*. Geographic variation in the intensity of petal reflectance is governed by temperature while pollinator-perceived color is governed by population structure established through post-glacial migration. Because these results in *C. americana* are correlational, additional tests of whether petal reflectance shows similar patterns across temperature gradients and examinations of how historical migration has influenced color evolution in more taxa will be important for understanding drivers of large scale biogeographic structuring in flower color.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MK and LG conceptualized the study. MK collected and analyzed the data. LG provided feedback on data analyses and interpretation. MK wrote the manuscript with input from LG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00991/full#supplementary-material

REFERENCES

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Asen, S., Stewart, R. N., and Norris, K. H. (1972). Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry* 11, 1139–1144. doi: 10.1016/S0031-9422(00)88467-8

Baranzelli, M. C., Johnson, L., Casacov, A., and Sérsic, A. N. (2014). Historical and ecological divergence among populations of *Monttea chilensis* (Plantaginaceae), an endemic endangered shrub bordering the Atacama Desert, Chile. *Evolution. Ecol.* 28, 751–744. doi: 10.1007/s10682-014-9694-y

Barnard-Kubow, K. B., Debban, C. L., and Galloway, L. F. (2015). Multiple glacial refugia lead to genetic structuring and the potential for reproductive isolation in a herbaceous plant. *Am. J. Bot.* 102, 1842–1853. doi: 10.3732/ajb.1500267

Berardi, A. E., Fields, P. D., Abbate, J. L., and Taylor, D. R. (2016). Elevational divergence and clinal variation in floral color and leaf chemistry in *Silene vulgaris*. *Am. J. Bot.* 103, 1508–1523. doi: 10.3732/ajb.1600106

Brunet, J. (2009). Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations with floral traits. *Ann. Bot.* 103, 1567–1578. doi: 10.1093/aob/mcp096

Caruso, C. M., Scott, S. L., Wray, J. C., and Walsh, C. A. (2010). Pollinators, herbivores, and the maintenance of flower color variation: a case study with Lobelia siphilitica. *Int. J. Plant Sci.* 171 (9), 1020–1028. doi: 10.3732/ajb.1300180

Chalker-Scott, L. (1999). Environmental Significance of Anthocyanins in Plant Stress Responses. *Photochem. Photobiol.* 70, 1–9. doi: 10.1111/j.1751-1097.1999.tb01944.x

Coberly, L. C., and Rausher, M. D. (2003). Analysis of a chalcone synthase mutant in *Ipomoea pur*purea reveals a novel function for flavonoids: amelioration of heat stress. *Mol. Ecol.* 12, 1113–1124. doi: 10.1046/j.1365-294X.2003.01786.x

Corbet, S. A., and Huang, S.-Q. (2016). Small bees overheat in sunlit flowers: do they make cooling flights? *Ecol. Entomol.* 41, 344–350. doi: 10.1111/een.12307

del Valle, J. C., Buide, M. L., Casimiro-Soriguer, I., Whittall, J. B., and Narbona, E. (2015). On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (Silene littorea). *Front. Plant Sci.* 6, 939. doi: 10.3389/fpls.2015.00939

Dick, C. A., Buenrostro, J., Butler, T., Carlson, M. L., Kliebenstein, D. J., and Whittall, J. B. (2011). Arctic Mustard Flower Color Polymorphism Controlled by Petal-Specific Downregulation at the Threshold of the Anthocyanin Biosynthetic Pathway. *PloS One* 6. doi: 10.1371/journal.pone.0018230

Galloway, L. F., Etterson, J. R., and Hamrick, J. L. (2003). Outcrossing rate and inbreeding depression in the herbaceous autotetraploid, *Campanula americana*. *Heredity* 90, 308–315. doi: 10.1038/sj.hdy.6800242

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. *PNAS* 98, 6253–6255. doi: 10.1073/pnas.111162598

Guitián, J. A., Sobral, M., Veiga, T., Losada, M., Guitián, P., and Guitián, J. M. (2017). Differences in pollination success between local and foreign flower color phenotypes: a translocation experiment with *Gentiana lutea* (Gentianaceae). *PeerJ* 5, e2882. doi: 10.7717/peerj.2882

Hallatschek, O., and Nelson, D. R. (2008). Gene surfing in expanding populations. *Theor. Population Biol.* 73, 158–170. doi: 10.1016/j.tpb.2007.08.008

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., and Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *Int. J. Climatol.* 25, 1965–1978. doi: 10.1002/joc.1276

Hopkins, R., Levin, D. A., and Rausher, M. D. (2012). Molecular signatures of selection on reproductive character displacement of flower color in *Phlox drummondii*. *Evolution* 66, 469–485. doi: 10.1111/j.1558-5646.2011.01452.x

Ison, J. L., Tuan, E. S. L., Koski, M. H., Whalen, J. S., and Galloway, L. F. (2019). The role of pollinator preference in the maintenance of pollen colour variation. *Ann. Bot.* 951–960. doi: 10.1093/aob/mcy211

Jorgensen, T. H., Richardson, D. S., and Andersson, S. (2006). Comparative analyses of population structure in two subspecies of *Nigella degenii*: evidence for diversifying selection on pollen-color dimorphisms. *Evolution* 60, 518. doi: 10.1554/05-393.1

Koski, M. H., and Ashman, T.-L. (2015). Floral pigmentation patterns provide an example of Gloger's rule in plants. *Nat. Plants* 1, 14007. doi: 10.1038/nplants.2014.7

Koski, M. H., and Galloway, L. F. (2018). Geographic variation in pollen color is associated with temperature stress. *New Phytol.* 218, 370–379. doi: 10.1111/nph.14961

Koski, M. H., Grossenbacher, D. L., Busch, J. W., and Galloway, L. F. (2017). A geographic cline in the ability to self-fertilize is unrelated to the pollination environment. *Ecology* 98, 2930–2939. doi: 10.1002/ecy.2001

Koski, M. H., Ison, J. L., Padilla, A., Pham, A. Q., and Galloway, L. F. (2018). Linking pollinator efficiency to patterns of pollen limitation: small bees exploit the plant–pollinator mutualism. *Proc. R. Soc B.* 285, 20180635. doi: 10.1098/rspb.2018.0635

Koski, M. H., Layman, N. C., Prior, C. J., Busch, J. W., and Galloway, L. F. (2019). Selfing ability and drift load evolve with range expansion. *Evol. Lett.* 3, 500–512. doi: 10.1002/evl3.136

Koski, M. H., Berardi, A. E., and Galloway, L. F. (2020). Pollen colour morphs take different paths to fitness. *J. Evolution. Biol.* 33, 388–400. doi: 10.1111/jeb.13599

Lacey, E. P., and Herr, D. (2005). Phenotypic plasticity, parental effects, and parental care in plants? I. An examination of spike reflectance in *Plantago lanceolata* (Plantaginaceae). *Am. J. Bot.* 92, 920–930. doi: 10.3732/ajb.92.6.920

Lacey, E. P., Lovin, M. E., Richter, S. J., and Herington, D. A. (2010). Floral Reflectance, Color, and Thermoregulation: What Really Explains Geographic Variation in Thermal Acclimation Ability of Ectotherms? *Am. Nat.* 175, 335–349. doi: 10.1086/650442

Lau, J. A., and Galloway, L. F. (2004). Effects of low-efficiency pollinators on plant fitness and floral trait evolution in *Campanula americana* (Campanulaceae). *Oecologia* 141, 577–583. doi: 10.1007/s00442-004-1677-1

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: New tools for the spectral and spatial analysis of colour in r. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Menzel, R., Steinmann, E., Souza, J. D., and Backhaus, W. (1988). Spectral Sensitivity of Photoreceptors and Colour Vision in the Solitary Bee, *Osmia rufa*. *J. Exp. Biol.* 136, 35–52.

Norgate, M., Boyd-Gerny, S., Simonov, V., Rosa, M. G. P., Heard, T. A., and Dyer, A. G. (2010). Ambient Temperature Influences Australian Native Stingless Bee (*Trigona carbonaria*) Preference for Warm Nectar. *PloS One* 5, e12000. doi: 10.1371/journal.pone.0012000

R Core Team (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/.

Rausher, M. D. (2008). Evolutionary Transitions in Floral Color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower color in the desert annual linanthus parryae: was Wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Shrestha, M., Garcia, J. E., Bukovac, Z., Dorin, A., and Dyer, A. G. (2018). Pollination in a new climate: Assessing the potential influence of flower temperature variation on insect pollinator behaviour. *PloS One* 13. doi: 10.1371/journal.pone.0200549

Skorupski, P., and Chittka, L. (2010). Photoreceptor Spectral Sensitivity in the Bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). *PloS One* 5, e12049. doi: 10.1371/journal.pone.0012049

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Sobral, M., Veiga, T., Domínguez, P., Guitián, J. A., Guitián, P., and Guitián, J. M. (2015). Selective Pressures Explain Differences in Flower Color among *Gentiana lutea* Populations. *PloS One* 10. doi: 10.1371/journal.pone.0132522

Stiles, E. A., Cech, N. B., Dee, S. M., and Lacey, E. P. (2007). Temperature-sensitive anthocyanin production in flowers of *Plantago lanceolata*. *Physiol. Plantarum* 129, 756–765. doi: 10.1111/j.1399-3054.2007.00855.x

Strauss, S., and Whittall, J. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and evolution of flowers*. Eds. L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press). 120–138.

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (*Anemone pavonina*, Ranunculaceae) in Northern Greece. *J. Comparat. Physiol. A* 205, 735–743. doi: 10.1007/s00359-019-01360-2

Streisfeld, M. A., and Kohn, J. R. (2005). Contrasting Patterns of Floral and Molecular Variation Across a Cline in *Mimulus aurantiacus*. *Evolution* 59, 2548–2559. doi: 10.1111/j.0014-3820.2005.tb00968.x

Streisfeld, M. A., and Kohn, J. R. (2007). Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evolution. Biol.* 20, 122–132. doi: 10.1111/j.1420-9101.2006.01216.x

Stuart-Fox, D., Newton, E., and Clusella-Trullas, S. (2017). Thermal consequences of colour and near-infrared reflectance. *Philos. Trans. R. Soc. B.: Biol. Sci.* 372, 20160345. doi: 10.1098/rstb.2016.0345

Thairu, M. W., and Brunet, J. (2015). The role of pollinators in maintaining variation in flower colour in the Rocky Mountain columbine, *Aquilegia coerulea*. *Ann. Bot.* 115, 971–979. doi: 10.1093/aob/mcv028

Thakur, P., Kumar, S., Malik, J. A., Berger, J. D., and Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: An overview. *Environ. Exp. Bot.* 67, 429–443. doi: 10.1016/j.envexpbot.2009.09.004

Vaidya, P., McDurmon, A., Mattoon, E., Keefe, M., Carley, L., Lee, C.-R., et al. (2018). Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (*Boechera stricta*). *New Phytol.* 218, 380–392. doi: 10.1111/nph.14998

van der Kooi, C. J., Kevan, P. G., and Koski, M. H. (2019). The thermal ecology of flowers. *Ann. Bot.* 124, 343–353. doi: 10.1093/aob/mcz073

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Willi, Y., Fracassetti, M., Zoller, S., and Van Buskirk, J. (2018). Accumulation of Mutational Load at the Edges of a Species Range. *Mol. Biol. Evol.* 35, 781–791. doi: 10.1093/molbev/msy003

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Flower Conspicuousness to Bees Across Pollination Systems: A Generalized Test of the Bee-Avoidance Hypothesis

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Flower signals of bee- and bird-pollinated plants have converged *via* pollinator-mediated evolution, driven by the visual system of their respective pollinators. For bird flowers, sensory exclusion of less effective bees is also important and such exclusion is also mediated by floral morphological filters. Likewise, other systems based on pollination by red-sensitive insects are also associated with red flowers displaying lower short-wavelength secondary peaks of reflectance, which decreases detectability to animals that are less sensitive to red, such as bees. These flowers often also present long tubes. Here, we tested a generalization of the bee-avoidance hypothesis in order to assess if it holds only for bird flowers or for other non-bee pollination systems as well. For this, we compared flower contrasts and spectral purity in bee visual systems as proxies for conspicuousness among four kinds of pollination systems: bee-visited flowers, insect-visited flowers (including bees and other insects), non-bee insect flowers (flowers visited by red-sensitive insects such as flies, butterflies and beetles, but not bees), and bird-visited flowers. We also assessed the association between conspicuousness to bees and flower depth, used as a proxy for morphological exclusion of bees. Overall, flower conspicuousness to bees differed only between insect (all three groups) and bird flowers, due to lower visual signals for the latter. This suggests that bee sensory exclusion *via* color signals is exclusive to bird flowers, while non-bee insect flowers might use other sensory channels to exclude bees, such as olfactory signals. Visual bee avoidance might be a mechanism exclusive to plants pollinated by specific guilds of red-sensitive insects not well represented in our sample. We also found a negative association between flower conspicuousness to bees and flower depth, suggesting an interplay of morphological and spectral traits in discouraging bee visits. Our results support the bee-avoidance hypothesis exclusively for bird flowers and an overall association between lower visual signals to bees and long tubes.

Keywords: pollination, bee avoidance, flower color, plant-animal communication, antagonism, flower depth, contrast, purity

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INTRODUCTION

Pollinator-mediated evolution of flower traits has been shown to be a major factor shaping angiosperm diversity [e.g., Schemske and Bradshaw (1999); Schiestl and Johnson (2013)]. Each pollinator group (e.g., bees, flies, and butterflies) responds to signals according to its, innate or not, preferences and sensorial skills, driving convergent flower evolution (Schiestl and Johnson, 2013). Floral color signals are expected to converge when sharing a specific guild of pollinators, such as UV-reflection gradients in bee flowers (Papiorek et al., 2016) and red reflection in bird flowers (Burd et al., 2014). Moreover, floral colors have often evolved in multiple-receiver contexts (Renoult et al., 2014). Nevertheless, consideration of the distinct pressures driven by multiple receivers, including less effective floral visitors, has been overlooked. Therefore, the contribution of pollinator preferences driving floral signal evolution may be overestimated in relation to selective pressures exerted by other visitors. Broad-scale comparisons of floral color considering less effective floral visitors may reveal unnoticed signaling patterns, with implications to the understanding of flower trait evolution.

Most bee flowers present inflection points (parts of rapidly changing reflectance that are optimally detected by visual systems in general) at regions of the spectrum of maximum discrimination for Hymenopteran vision [Chittka (1992); Dyer et al. (2012)], indicating a loose signal-receptor match that enhances detectability by bees. The same match has been found for bird-flower signals and bird vision (Shrestha et al., 2013). In the case of plants pollinated by hummingbirds, bee vision seems to have played a critical role as well, likely due to negative consequences of bee visitors in bird flowers (Bergamo et al., 2019). Bee pollination is known to precede vertebrate pollination in evolutionary history (Rosas-Guerrero et al., 2014), and bee-bird pollinator shifts may have occurred because of the differential associations these vectors have with pollen: while bees use it as a resource to feed their larvae, birds mostly ignore it (Thomson and Wilson, 2008). Moreover, vertebrate pollinators like birds require more energy and have the potential to transfer pollen at longer distances than bees (Ashworth et al., 2015) presumably because of their increased mobility, size and energy requirements (Rosas-Guerrero et al., 2014). Thus, selective pressures on bird flowers to deter bees from using pollen and depleting the costly, copious nectar required by this new type of pollen vector are expected (Thomson and Wilson, 2008).

The negative effects of bees on bird flowers lays the foundation for the **bee-avoidance hypothesis** (Raven, 1972), which attempts to explain an apparently unreasonable number of red bird flowers. Reddish colors are less detectable by bees, since these insects exhibit low red-wavelength sensitivity (Lunau et al., 2011). As a result, birds would visit more frequently red flowers in order to avoid competition with bees (Rodríguez-Gironés and Santamaría, 2004), the most abundant anthophilous animals (Michener, 2000). Bird flowers, in their turn, were selected to display colors less discriminable by bees, like red, imposing enough additional foraging costs for discouraging bee visits [Raven (1972); Lunau et al. (2011)], even though bees are not completely blind to red light (Chittka and Waser, 1997). In this case, pollinator preference is unlikely because birds visit flowers of other colors (Papiorek et al., 2016) and present no innate preference for red (Lunau et al., 2011). Bird flowers also lack known olfactory signals and present long tubes that may act as filters against bees since foraging costs for these insects increase with flower depth (Harder and Cruzan, 1990). However, it is unclear if flower depth is negatively associated with conspicuousness to bees across the angiosperms. All these traits seem to work synergistically, creating not just filters but a private channel of communication between birds and bee-avoiding, odorless, long-wavelength-reflecting and long-tubed bird flowers [Castellanos et al. (2004); Willmer (2011); Gegear et al. (2017)]. The bee-avoidance hypothesis for bird-pollinated plants has been hitherto supported by case studies [e.g., Bergamo et al. (2016); Gegear et al. (2017)] and broader comparisons between bee and bird flowers [e.g., Lunau et al. (2011); Camargo et al. (2019)].

Bees are the most diverse and abundant group of floral visitors in most ecosystems (Michener, 2000). Therefore, bees could have influenced the evolution of any animal-pollinated system. Little is known about pollinator shifts between insect groups, like bee-fly shifts, or how non-bee insect systems have evolved in the presence of bees. In other words, it is uncertain if visual bee-avoidance mechanisms have evolved in other non-bee systems. For Australian orchids, flower color was found to differ between bee- and fly-pollinated species, the latter having most of their inflection points beyond 500 nm (Shrestha et al., 2019), as found for bee-avoiding bird flowers (Lunau et al., 2011), which is roughly the limit of bee chromatic vision. The same difference was found between flowers of a community with a dipteran-exclusive pollinator fauna in Macquarie Island and their inland bee-pollinated relatives (Shrestha et al., 2016). In both cases, fly flowers seem to be more constrained in bee visual color space and less spectrally diverse than bee-flower color, which seems to be a general feature of these flowers Willmer (2011). Nevertheless, if non-bee insect flowers are less conspicuous to bee vision remains to be tested. Furthermore, red flowers are sometimes associated with other insect pollination systems such as butterfly- [e.g., Johnson and Bond (1994); Willmer (2011)] and beetle-pollinated species (Dafni et al., 1990). These differ from bees in their visual systems presenting a higher red sensitivity, which was demonstrated for some species [e.g., flies: Lunau (2014); beetles, butterflies: Briscoe and Chittka (2001)].

Red flowers pollinated by insects with red receptors have been shown to present lower reflectance intensity of secondary peaks at shorter wavelengths (Chen et al., 2020), which might result in lower contrasts to animals lacking red receptors like bees. Thus, we hypothesize that bee vision might be a factor acting on flower color selection in non-bee insect-pollinated systems, resulting in lower contrasts that might act synergistically with long tubes also found for butterfly- Willmer (2011) and some fly-pollinated (Goldblatt and Manning, 2000) species.

We tested a generalization of the bee-avoidance hypothesis in a broad-scale set of species, using plants spread on the phylogeny of angiosperms, which have evolved in diverse geographical and ecological backgrounds. Using flower reflectance data, we computed three metrics for conspicuousness in the bee visual

systems. We then compared the conspicuousness of species visited by bees to those of species visited by other groups to test whether all non-bee-pollinated systems have evolved bee-avoidance mechanisms. We expected lower conspicuousness for species visited by other groups in comparison to species visited by bees (i.e., a general bee-avoidance pattern). We also looked for an association between flower depth and conspicuousness regardless of pollination system, to test if long tubes and low conspicuousness to bees are key traits that act synergistically in long-tubed flowers.

MATERIAL AND METHODS

Species Sample and Study Area

Data for 233 out of the total 389 species (see **Table S1** for the complete species list and **Table S2** for data sources) in our sample were downloaded from the Floral Reflectance Database [hereafter "FReD", Arnold et al. (2008); accessed in August 2019]. We included all species for which reflectance, flower depth and flower visitors' identity were available. Additionally for these species, leaf reflectance was also downloaded when available. The remaining 156 species were sampled at the Botanical Garden of Rio de Janeiro, Brazil (hereafter "JBRJ"). Flowers were sampled from February 2017 to August 2019 at the arboretum, a live collection house to roughly nine-thousand species in its 54 ha (http://rb.jbrj.gov.br/arboreto/arboretoleaflet.php). For all animal-pollinated species in bloom, at least three samples of the most dominant attractive structure in the display of a given species (see *Spectral Reflectance*) and three leaves were collected according to availability. Following literature research on visitors' data for each plant species sampled at JBRJ, two final datasets were created. The first one, used for comparisons between pollination systems, comprised 285 species (233 from FReD and 52 from JBRJ). This group of species had available information on flower visitors which fell in the pollination system categories established, thus we excluded species visited by underrepresented groups (see *Pollination Systems*). We also excluded species for which we found evidence of sexual deceit and sapromyophily, since these flower colors evolved in different contexts from those where bee-avoidance mechanisms would be expected. The second dataset, used for assessing the association between bee contrast and flower depth, included all species but those with dish-shaped flowers (see *Flower Depth Measurements*), totaling 286 species (135 from FReD and 151 from JBRJ).

Spectral Reflectance

Reflectance measurements of each structure (five on average) of species sampled at the Botanical Garden of Rio de Janeiro were taken using a portable spectrometer (USB 4000; Ocean Optics) at an angle of 45°. Barium sulfate ($BaSo_4$) was used as white standard and a black chamber as black standard [Lunau et al. (2011); Bergamo et al., 2016)]. Only the predominant color in the display of a given species was considered (see **Table S2** for a list of structures). We decided to include structures such as bracts and calyces because bracts were found to play a more important role in bee avoidance than petals in bracted species (Bergamo et al., 2019). We hereafter refer to all structures generally as "flowers". We restricted the analyses to the 300–700 nm wavelength range, which falls under the spectral sensitivity of animal pollinators.

Flower Color Categories

We classified flower reflectance spectra of each species into color categories based on their average reflectances in the UV, blue, green, and red wavebands, following Camargo et al. (2019) and Chittka et al. (1994) but modifying the thresholds between absorbance and reflectance according to the distribution of our data (**Figure 1**, see **Table S2** for mean intensity values in each band and **Figure S1** for the mean reflectance curve of the whole dataset). In order to discriminate absorbance (−) from reflectance (+) in a given waveband, we used as thresholds 10% for the UV, 30% for the blue, 40% for the green, and 60% for the red bands. Additionally, we classified flowers reflecting in the green band with a difference ≥ 50% in relation to the mean reflection in the blue/red bands as green-absorbing [adapted from Camargo et al. (2019), see **Figure S2** for the mean reflectance curves of each color category].

Flower Depth Measurements

The total flower depth of on average five (but at least three) flowers of the species sampled at the Botanical Garden of Rio de Janeiro was measured with a digital caliper. For all other species, data were extracted directly from FReD [Arnold et al. (2008); accessed in August 2019]. We included in our dataset for flower depth analysis species for which flower visitors were unknown. When flower visitors' identity was available, however, we excluded species whose visitors did not fall into the categories established for comparisons across pollination systems (see *Pollination Systems*). Since we aimed at investigating the interplay between two bee-avoiding traits, namely long flower tubes and low conspicuousness, we excluded dish-shaped flowers from this analysis, in practice considering only species with flower *depth* ≥ 1 mm. We then applied log scale for normalization of flower depth data.

Pollination Systems

Data for floral visitors of each species sampled at JBRJ were obtained from the literature using as key words "pollinat* + [name of the species]". Since these sources followed different methodologies, we considered these data at the visitor level as potential pollinators. We did not take into account any morphological features (i.e., pollination syndromes). We acknowledge the limitations of using visitors' data rather than pollination effectiveness for defining groups. However, since our main focus was on the role of antagonists in trait selection, we believe this is unlikely to affect our results. Thus, four groups of species were designated according to their visitors' identity as distinct pollination systems (**Figure 2**, see **Table S3**): bee flowers (137 species visited solely by bees), bee+insect flowers (90 species visited by bees and other insects), non-bee insect flowers (26 species visited by potentially red-sensitive insects, especially flies but also beetles and butterflies or any combination between them; see **Figure 2**) and bird flowers (32 species visited solely by birds). Thus, we have included both functionally specialized

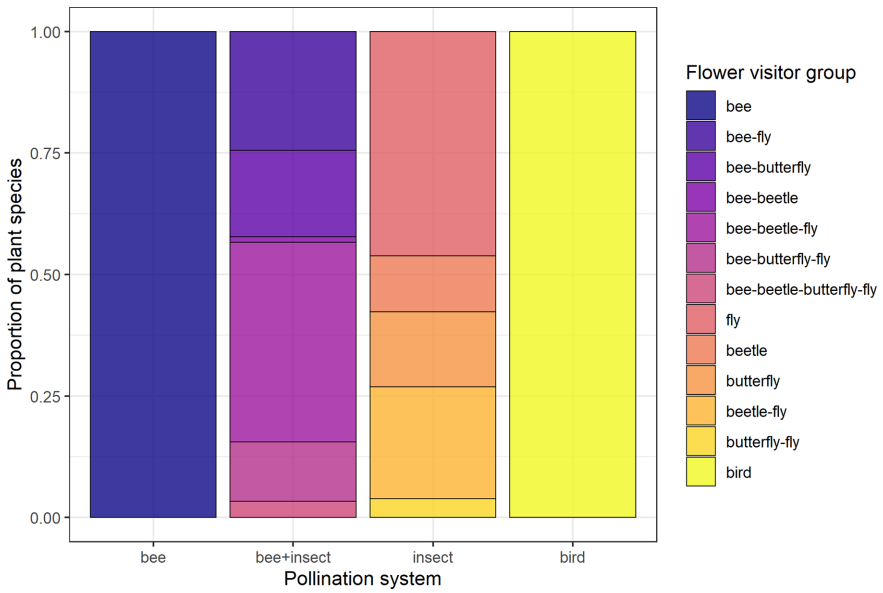


FIGURE 1 | Composition of the four groups of pollination systems designated according to flower visitor identity of each species. Groups are: "Bee" (species visited solely by bees, *N* = 137), "bee+insect" (species visited by both bees and other insects, *N* = 90), "insect" (non-bee insect flowers, i.e., species visited by potentially red-sensitive insects, but not by bees; *N* = 26) and "bird" (species visited solely by birds, *N* = 32).

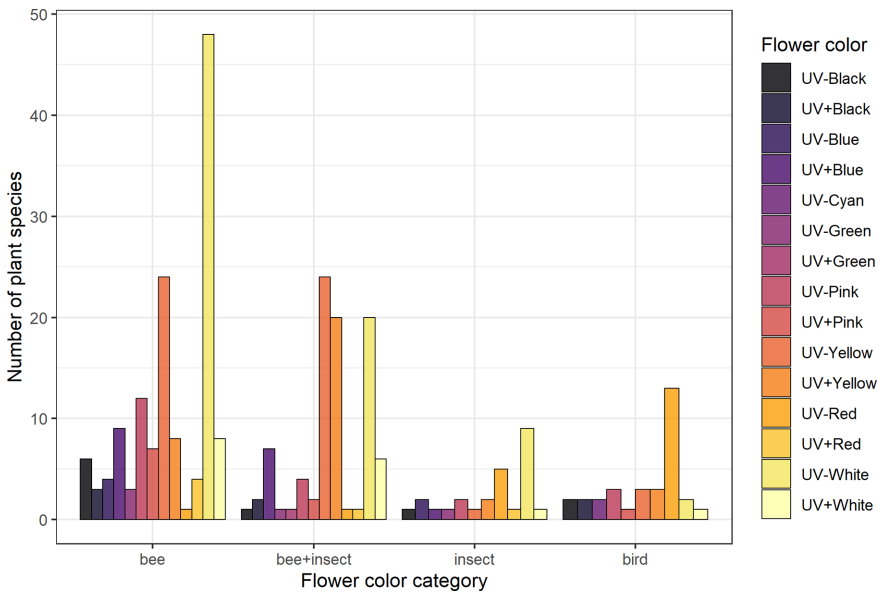


FIGURE 2 | Frequency of flower color categories for each pollination system considered in the analyses. Groups are: "Bee" (species visited solely by bees, *N* = 137), "bee+insect" (species visited by both bees and other insects, *N* = 90), "insect" (non-bee insect flowers, i.e., species visited by potentially red-sensitive insects, but not by bees; *N* = 26) and "bird" (species visited solely by birds, *N* = 32). Flower color categorization was done by averaging reflectance intensity across four bands of the spectrum: UV (from 201 to 300 nm), blue (301–400 nm), green (401–500 nm), and red (501–600 nm) and then assigning either "absorbing" (–) or "reflecting" (+) for each band according to thresholds selected based on the distribution of the data. See *Flower Color Categories* and **Table S2** for details.

(pollinated solely by one animal group) and generalized pollination systems [*sensu* Ollerton et al. (2007)].

Color Conspicuousness to Bee Vision

Using the mean reflectance curves of each species, three parameters of color conspicuousness to bees were calculated: achromatic contrast against the background (*ACB* hereafter), chromatic contrast against the background (*CCB*) and spectral purity (*SP*). These were computed according to photoreceptor spectral sensitivities available for the model bee species *Apis mellifera* L. and *Bombus terrestris* L. (Peitsch et al., 1992). We chose those species because honeybees and bumblebees are important pollinators in several ecosystems (Michener, 2000).

We defined *CCB* as the distance between the loci of the flower and the background (Rohde et al., 2013) in the color hexagon of Chittka (1992) and *ACB* (or "green contrast") as the contrast produced by the green photoreceptor between the stimulus and the background (Rohde et al., 2013). Both of these metrics are of importance because bees use chromatic cues at shorter distances and achromatic cues at longer ones, depending on visual angle (Spaethe et al., 2001). Finally, *SP* refers to the saturation of a given color and is relevant because bees have been shown to prefer colors of high spectral purity when foraging [Lunau et al. (1996); Rohde et al. (2013)]. We used the mean reflectance of all leaves in our sample as the standard leaf background for all species [following Renoult et al. (2015), see **Figure S1** for the mean leaf reflectance used and **Table S4** for all contrast values] and a standard daylight function (D65 irradiance function) as illuminant in the vision models. Using an alternative forest-shade illuminance function did not qualitatively affect our results because we used von Kries correction, which assumes that bee receptors adapt to these changes in illuminant. Even though we recognize the limitations of our standardized approach for species that evolved in diverse illuminants backgrounds, we note that common parameters are necessary for broad-scale comparisons since no specific background and illuminance reflectances were available for each plant species in our dataset. All visual modeling was done with the "pavo" package (Maia et al., 2019) in R software (R Core Team, 2013).

Statistical Analyses

In order to assess the sensorial exclusion of bees in non-bee flowers, we compared mean contrasts (*ACB* and *CCB*) of bee flowers to those of flowers visited by other vectors, i.e., different pollination systems (bee+insect; non-bee insect, and bird flowers). We ran separate ANOVA tests each using a contrast measurement as the response variable and pollination systems as the explanatory one (four models in total: *ACB* and *CCB* for both *Apis* and *Bombus* visual systems). Then, we computed *post-hoc* Tukey HSD tests to identify the pairs of significant differences between the four kinds of pollination system.

For the analysis of association between flower depth and bee contrast, we fitted four separate linear regressions using flower depth as the explanatory variable and each of the contrast measurements as response variables (Bergamo et al., 2019). Log scale was applied to flower depth for normalization.

To verify if bee contrasts against the background (*ACB* and *CCB*) would be meaningful even against a background of high purity, we verified their relationship with spectral purity (*SP*) in a linear regression, which showed a positive association (**Figure S3**).

In order to account for phylogenetic signal in our sample, we built a phylogenetic hypothesis using the PhytoPhylo megaphylogeny [Qian and Jin (2016) modified from Zanne et al. (2014)] in R for the full dataset (comprising all 389 species). Three different trees for different scenarios were generated, according to choices as to how to insert the branches not found in the megaphylogeny [see Qian and Jin (2016) for details]. The phylogenetic signal of all contrast variables and of flower depth were calculated as Blomberg's K (Blomberg et al., 2003) using the *phylosig()* function of the "phytools" R-package (Revell, 2012). Values of $K < 1$ indicate that there is no or little phylogenetic signal for that trait (Blomberg et al., 2003). None of the contrasts nor flower depth showed evidence for phylogenetic signal (K close to 0 in all cases, range 0.01–0.09; see **Table S5**).

RESULTS

Visual modeling of spectral reflectance data yielded *ACB* values ranging from 0.01 to 0.43 for *Apis* (0.26 ± 0.11; mean ± SD hereafter) and from 0.01 to 0.42 for *Bombus* models (0.25 ± 0.11); and *CCB* values ranging from 0.01 to 0.25 in the *Apis* model (0.14 ± 0.06) and from 0.01 to 0.34 in the *Bombus* model (0.18 ± 0.08). Flower depth values ranged from 0.00 mm to 89.37 mm (12.49 ± 16.71 mm; see **Table S4**).

Conspicuousness Across Pollination Systems

We found a significant effect of pollination system ($p < 0.01$ in all models, see **Table S6**) for both visual models in the achromatic (ACB_{Apis}: $F = 12.25$; ACB_{Bombus}: $F = 12.44$), chromatic channels (CCB_{Apis}: $F = 23.83$; CCB_{Bombus}: $F = 21.62$) and spectral purity (SP_{Apis}: $F = 19.95$; SP_{Bombus}: $F = 17.75$).

Overall, only the bird-flower group differed from the others in mean contrast against the background (**Figure 3**, see **Table S7**). Insect-flower groups (bee, non-bee insect, and bee+insect flowers) did not differ between them in most models, except in CCB_{Apis} between bee and bee+insect flowers (**Figure 3**, third panel). We found significantly lower *ACB*, *CCB*, and *SP* for bird flowers in relation to all groups of insect flowers in both visual models ($p < 0.01$ for all models).

Conspicuousness Comparisons Across Flower Color Categories

UV-absorbing red flowers presented the lowest contrast and purity values, while UV-absorbing white flowers were the most conspicuous ones in the eyes of bees (**Figure 4**; see **Table S8**). UV-reflecting and UV-absorbing yellow flowers did not differ in bee contrast in any of the models (but differed in one model for spectral purity), and presented lower contrasts and purity than

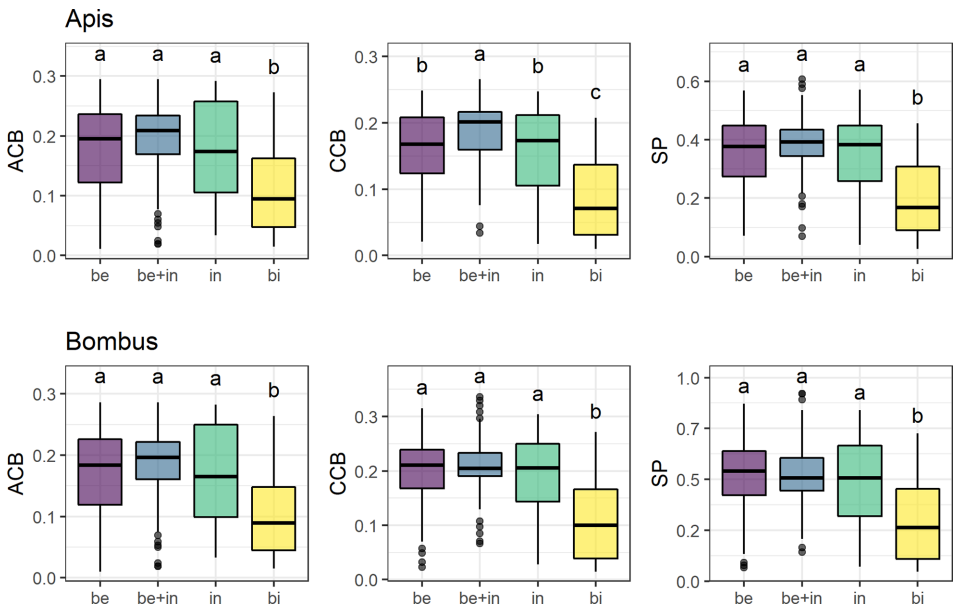


FIGURE 3 | Mean achromatic (*ACB*) and chromatic (*CCB*) contrast against the background and spectral purity (*SP*) comparisons between flowers of 285 species belonging to four distinct pollination systems in the visual models for *Apis mellifera* and *Bombus terrestris*. Groups are: "Be" (species visited solely by bees, *N* = 137), "be+in" (species visited by both bees and other insects, *N* = 90), "in" (non-bee insect flowers, i.e., species visited by potentially red-sensitive insects, but not by bees; *N* = 26) and "bi" (species visited solely by birds, *N* = 32). Box-plots show the distribution of contrast and purity values for each pollination system: the thick horizontal line indicates the median; lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the smallest/largest value at most 1.5 * *IQR* of the hinge and individual dots indicate outliers. Different letters represent significant differences between means after ANOVA and *post-hoc* Tukey's HSD tests.

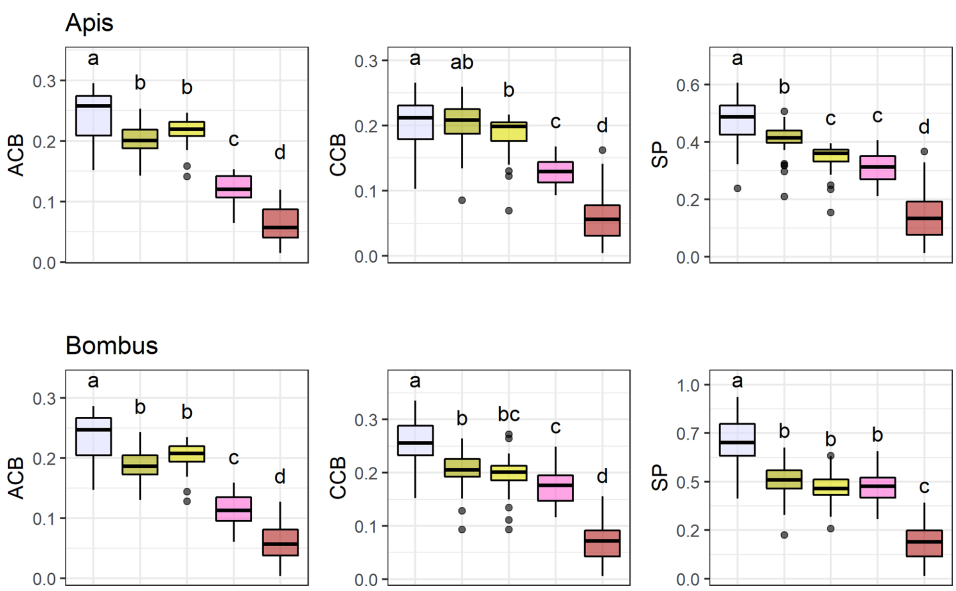


FIGURE 4 | Mean achromatic (*ACB*) and chromatic (*CCB*) contrast against the background and spectral purity (*SP*) comparisons between the most frequent flower color categories. Color categories are UV-absorbing white (*N* = 9 7 species), UV-absorbing yellow (*N* = 63), UV-reflecting yellow (*N* = 37), UV-absorbing pink (*N* = 31), and UV-absorbing red (*N* = 55). Only colors with *N* > 30 were considered (totaling 288 species). Flower contrasts and spectral purity were computed for *Apis mellifera* and *Bombus terrestris*. Box-plots show the distribution of contrast and purity values for each pollination system: the thick horizontal line indicates the median; lower and upper hinges correspond to the first and third quartiles; whiskers extend from the hinge to the smallest/largest value at most 1.5 * *IQR* of the hinge and individual dots indicate outliers. Different letters represent significant differences between means after ANOVA and *post-hoc* Tukey's HSD tests.

UV-absorbing white flowers in most models, but higher ones than UV-absorbing red flowers as did UV-absorbing pink flowers. Not surprisingly, UV-absorbing red flowers were the most frequent in the bird pollination system, whereas most bee and non-bee insect flowers were UV-absorbing white (**Figure 4**).

Association Between Conspicuousness and Flower Depth

We found a negative relationship between conspicuousness to bees and flower depth ($p < 0.01$) for all models (**Figure 5**, see **Table S9**), indicating that flower contrasts against the background decrease with flower depth in both achromatic (ACB_{Apis} and ACB_{Bombus}: $R^2 = 0.09$) and chromatic channels (CCB_{Apis}: $R^2 = 0.15$; CCB_{Bombus}: $R^2 = 0.09$), as does spectral purity (SP_{Apis}: $R^2 = 0.12$; SP_{Bombus}: $R^2 = 0.07$).

DISCUSSION

Overall, the three pollination systems composed by insect visitors here studied seem to be hardly distinguishable in detectability properties to bee vision, for all three conspicuousness metrics used (i.e., *ACB*, *CCB*, and *SP*). This suggests that insect flowers use similar strategies in their intensity of visual signals detectable by bees. Two processes could account for these results. First, in spite of notable red-sensitive exceptions, an overall conservative visual system in flower-visiting insects with similar points of optimal discriminability in the spectrum [i.e., overall similar visual systems; Chittka (1996)], selecting similar patterns of reflectance. Second, bee visits could have positive effects (or at least not incur in costs) in insect flowers in general (Sampson et al., 2004). In any case, our results did not support the bee-avoidance hypothesis (Raven, 1972) through color signals for non-bee insect-pollinated species. Thus, if some kind of bee avoidance does occur in insect flowers, it may happen either only for specific systems with red flowers and based on specific guilds that were underrepresented in our sample, or in other sensory channels rather than the visual one. Unlike birds, insects use a great number of olfactory cues when foraging for floral resources, not relying solely on spectral signals (Andersson et al., 2015). Hence, plants that specialize in different insect pollinator groups dispose of a large spectrum of olfactory and even tactile, heat and electric signals, besides visual ones, which may be combined to create exclusive channels with their most effective pollinators [Wedzony and Filek (1998); Schiestl and Dötterl (2012); Telles et al. (2017)]. In this way, visual bee avoidance does not seem to be such a widespread phenomenon in insect-pollinated flowers as it is for bird-pollinated ones.

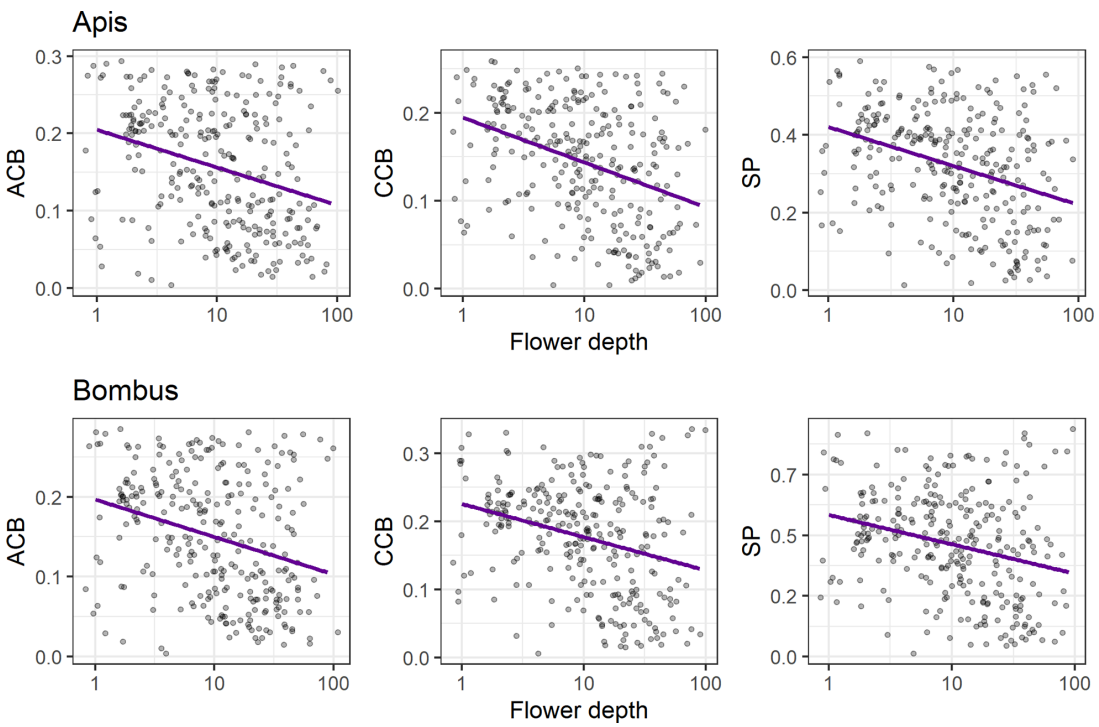


FIGURE 5 | Linear regression analyses of bee contrasts against the background (achromatic, *ACB*; chromatic, *CCB*) and spectral purity (*SP*) with flower depth (mm), for *Apis mellifera* (upper panels) and *Bombus terrestris (lower panels*). Left panels: negative associations between flower depth and *ACB* ($R^2 = 0.09$ and $p < 0.01$ for both *Apis* and *Bombus* models); central panels: negative associations between flower depth and *CCB* for the *Apis* model (upper panel; $R^2 = 0.15$ and $p < 0.01$) and for the *Bombus* model (lower panel; $R^2 = 0.08$ and $p < 0.01$); right panels: negative associations between flower depth and *SP* for the *Apis* model (upper panel; $R^2 = 0.12$ and $p < 0.01$) and for the *Bombus* model (lower panel; $R^2 = 0.07$ and $p < 0.01$). Only flowers with flower depth ≥ 1 mm were considered ($N = 286$ species), regardless of pollination system. We applied log scale to flower depth for normalization and horizontal jittering in order to prevent overplotting.

Nevertheless, bird flowers differed from bee flowers in our database with significantly lower mean contrasts and spectral purity, which gives additional support to the bee-avoidance hypothesis [Bergamo et al. (2019); Camargo et al. (2019)]. This is probably due to two factors. First, the high frequency of UV-absorbing red flowers, the least conspicuous ones, in the bird group, which was to be expected since most bird-specialized flowers are red [Grant (1966); Wilson et al. (2006)]. Moreover, the predominance of UV-absorbing white flowers in the bee group, which were the most conspicuous ones in our models. These results reinforce the role of reddish flowers and bracts in bee-avoiding bird-specialized species as a general pattern rather than a phenomenon restricted to specific communities.

We found a negative relationship between conspicuousness to bees and flower depth, similarly to the results of *CCB* in hummingbird flowers by Bergamo et al. (2019) and of *ACB* in insect flowers by Binkenstein et al. (2017). Our results indicate that the low conspicuousness of long-tubed flowers to bees might not be a phenomenon exclusive to bird-pollinated systems. Moreover, long-tubed flowers in our study presented low conspicuousness in both achromatic and chromatic channels, expanding previous results found for the chromatic channel in hummingbird flowers of the Brazilian Atlantic forest (Bergamo et al., 2019). Thereby, our results support that flower depth and detectability to bees are two of a set of key traits mediating bee avoidance in flowers (Gegear et al., 2017). These two traits might be under similar selective pressures, since long flower tubes are associated with increased nectar robbing by bees [Navarro and Medel (2009); Rojas-Nossa et al. (2016)], and bee nectar robbers have a strong negative effect on the reproductive success of hummingbird-pollinated flowers [Irwin et al. (2001); Bergamo and Sazima (2018)]. On the other hand, for long-tubed bee-pollinated flowers, lower contrasts and purity might signal nectar inaccessibility to short-tongued bees (and even to other insects with short mouthparts), decreasing foraging energy losses for the potential visitors and enhancing reproductive success for the plant through reduced interference of these with legitimate pollinators (Binkenstein et al., 2017). Moreover, total flower depth might be regarded as a proxy for area of attraction, since it is one way of determining overall flower size (Wolf et al., 1976). In theory, a species under selection for increased floral area of attraction may have smaller investment in pigments, which might cause a decrease in its contrast (Blarer et al., 2002). Such a trade-off might be at play in tubular flowers, yielding a negative association between flower size and bee contrast regardless of pollination system.

CONCLUSION

Our results corroborate the bee-avoidance hypothesis for bird flowers. However, for insect flowers, all groups of flowers presented a similar intensity of color signals to bees, giving no support to visual bee avoidance. Therefore, our results suggest that sensory exclusion of bees *via* color signals is a mechanism exclusive to bird-pollinated species. We also found a negative association between flower depth and conspicuousness in the visual models, regardless of pollination system, suggesting a general correlation between two bee-avoiding traits that may act synergistically. Further studies could enlighten the role of long corolla tubes in insect-bird pollinator shifts, investigating whether long-tubed species are more likely to shift to bird pollination. The possibility of visual bee-avoidance mechanisms should also be investigated for pollination systems based on specific non-bee insect guilds, especially those with long tubes, in communities where bees are also present. Overall, our results reinforce the importance of spectral signaling in bird-pollinated systems and its interplay with flower depth.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LF and MR-G devised the initial research project. GC and CA collected the data supervised by LF. GC conducted data analysis with support from PB and interpreted its findings along with LF. GC wrote the manuscript with assistance from LF, PB, and MR-G.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.558684/full#supplementary-material

REFERENCES

Andersson, M. N., Löfstedt, C., and Newcomb, R. D. (2015). Insect olfaction and the evolution of receptor tuning. *Front. Ecol. Evol.* 3, 53. doi: 10.3389/fevo.2015.00053

Arnold, S., Savolainen, V., and Chittka, L. (2008). FReD: the floral reflectance spectra database. *Nat. Precedings* 1–1. doi: 10.1038/npre.2008.1846.1

Ashworth, L., Aguilar, R., Martén-Rodríguez, S., Lopezaraiza-Mikel, M., Avila-Sakar, G., Rosas-Guerrero, V., et al. (2015). "Pollination syndromes: a global pattern of convergent evolution driven by the most effective pollinator," in *Evolutionary biology: biodiversification from genotype to phenotype* (Cham: Springer), 203–224.

Bergamo, P. J., and Sazima, M. (2018). Differential outcomes of nectar robbing on the reproductive success of a melittophilous and an ornithophilous species. *Int. J. Plant Sci.* 179, 192–197. doi: 10.1086/696234

Bergamo, P. J., Rech, A. R., Brito, V. L., and Sazima, M. (2016). Flower colour and visitation rates of Costus arabicus support the 'bee avoidance' hypothesis for red-reflecting hummingbird-pollinated flowers. *Funct. Ecol.* 30, 710–720. doi: 10.1111/1365-2435.12537

Bergamo, P. J., Wolowski, M., Telles, F. J., De Brito, V. L. G., Varassin, I. G., and Sazima, M. (2019). Bracts and long-tube flowers of hummingbird-pollinated plants are conspicuous to hummingbirds but not to bees. *Biol. J. Linn. Soc.* 126, 533–544. doi: 10.1093/biolinnean/bly217

Binkenstein, J., Stang, M., Renoult, J. P., and Schaefer, H. M. (2017). Weak correlation of flower color and nectar-tube depth in temperate grasslands. *J. Plant Ecol.* 10, 397–405. doi: 10.1093/jpe/rtw029

Blarer, A., Keasar, T., and Shmida, A. (2002). Possible mechanisms for the formation of flower size preferences by foraging bumblebees. *Ethology* 108, 341–351. doi: 10.1046/j.1439-0310.2002.00778.x

Blomberg, S. P., Garland, J. T., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1111/j.0014-3820.2003.tb00285.x

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B: Biol. Sci.* 281, 20132862. doi: 10.1098/rspb.2013.2862

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Castellanos, M., Wilson, P., and Thomson, J. (2004). 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in Penstemon flowers. *J. Evol. Biol.* 17, 876–885. doi: 10.1111/j.1420-9101.2004.00729.x

Chen, Z., Niu, Y., Liu, C.-Q., and Sun, H. (2020). Red flowers differ in shades between pollination systems and across continents. *Ann. Bot.* doi: 10.1093/aob/mcaa103

Chittka, L., and Waser, N. M. (1997). Why red flowers are not invisible to bees. *Israel J. Plant Sci.* 45, 169–183. doi: 10.1080/07929978.1997.10676682

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L. (1996). Does bee color vision predate the evolution of flower color? *Naturwissenschaften* 83, 136–138. doi: 10.1007/BF01142181

Dafni, A., Bernhardt, P., Shmida, A., Ivri, Y., Greenbaum, S., O'Toole, C., et al. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. *Israel J. Plant Sci.* 39, 81–92. doi: 10.1080/0021213X.1990.10677134

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G., Simonov, V., and Wong, B. B. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B: Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Gegear, R. J., Burns, R., and Swoboda-Bhattarai, K. A. (2017). "hummingbird" floral traits interact synergistically to discourage visitation by bumble bee foragers. *Ecology* 98, 489–499. doi: 10.1002/ecy.1661

Goldblatt, P., and Manning, J. C. (2000). The long-proboscid fly pollination system in southern Africa. *Ann. Missouri Bot. Garden* 87, 146–170. doi: 10.2307/2666158

Grant, K. A. (1966). A hypothesis concerning the prevalence of red coloration in California hummingbird flowers. *Am. Nat.* 100, 85–97. doi: 10.1086/282403

Harder, L., and Cruzan, M. (1990). An evaluation of the physiological and evolutionary influences of inflorescence size and flower depth on nectar production. *Funct. Ecol.* 4, 559–572. doi: 10.2307/2389323

Irwin, R. E., Brody, A. K., and Waser, N. M. (2001). The impact of floral larceny on individuals, populations, and communities. *Oecologia* 129, 161–168. doi: 10.1007/s004420100739

Johnson, S., and Bond, W. (1994). "Red flowers and butterfly pollination in the fynbos of South Africa," in *Plant-animal interactions in Mediterranean-type ecosystems* (Dordrecht: Springer), 137– 148.

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 178, 477–489. doi: 10.1007/BF00190178

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K. (2014). Visual ecology of flies with particular reference to colour vision and colour preferences. *J. Comp. Physiol. A* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Michener, C. D. (2000). *The bees of the world* Vol. 1 (Baltimore: JHU press).

Navarro, L., and Medel, R. (2009). Relationship between floral tube length and nectar robbing in Duranta erecta L.(Verbenaceae). *Biol. J. Linn. Soc.* 96, 392–398. doi: 10.1111/j.1095-8312.2008.01146.x

Ollerton, J., Killick, A., Lamborn, E., Watts, S., and Whiston, M. (2007). Multiple meanings and modes: on the many ways to be a generalist flower. *Taxon* 56, 717–728. doi: 10.2307/25065855

Papiorek, S., Junker, R. R., Alves-dos Santos, I., Melo, G. A., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Qian, H., and Jin, Y. (2016). An updated megaphylogeny of plants, a tool for generating plant phylogenies and an analysis of phylogenetic community structure. *J. Plant Ecol.* 9, 233–239. doi: 10.1093/jpe/rtv047

R Core Team (2013). *R: A language and environment for statistical computing*. Vienna, Austria. Available at: https://www.R-project.org/.

Raven, P. H. (1972). Why are bird-visited flowers predominantly red? *Evolution* 26, 674–674. doi: 10.1111/j.1558-5646.1972.tb01975.x

Renoult, J. P., Valido, A., Jordano, P., and Schaefer, H. M. (2014). Adaptation of flower and fruit colours to multiple, distinct mutualists. *New Phytol.* 201, 678–686. doi: 10.1111/nph.12539

Renoult, J. P., Blüthgen, N., Binkenstein, J., Weiner, C. N., Werner, M., and Schaefer, H. M. (2015). The relative importance of color signaling for plant generalization in pollination networks. *Oikos* 124, 347–354. doi: 10.1111/oik.01361

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Rodríguez-Gironés, M. A., and Santamaría, L. (2004). Why are so many bird flowers red? *PLoS Biol.* 2, e350. doi: 10.1371/journal.pbio.0020350

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (Bombus terrestris) and honeybees (Apis mellifera) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Rojas-Nossa, S. V., Sánchez, J. M., and Navarro, L. (2016). Nectar robbing: a common phenomenon mainly determined by accessibility constraints, nectar volume and density of energy rewards. *Oikos* 125, 1044–1055. doi: 10.1111/oik.02685

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., et al. (2014). A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.* 17, 388–400. doi: 10.1111/ele.12224

Sampson, B. J., Danka, R. G., and Stringer, S. J. (2004). Nectar robbery by bees Xylocopa virginica and Apis mellifera contributes to the pollination of rabbiteye blueberry. *J. Econ. Entomol.* 97, 735–740. doi: 10.1603/0022-0493(2004)097[0735:NRBBXV]2.0.CO;2

Schemske, D. W., and Bradshaw, H. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus). *Proc. Natl. Acad. Sci.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Schiestl, F. P., and Dötterl, S. (2012). The evolution of floral scent and olfactory preferences in pollinators: coevolution or pre-existing bias? *Evolution* 66, 2042–2055. doi: 10.1111/j.1558-5646.2012.01593.x

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., and Burd, M. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Shrestha, M., Burd, M., Garcia, J., Dorin, A., and Dyer, A. (2019). Colour evolution within orchids depends on whether the pollinator is a bee or a fly. *Plant Biol.* 21, 745–752. doi: 10.1111/plb.12968

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Telles, F. J., Corcobado, G., Trillo, A., and Rodriguez-Girones, M. A. (2017). Multimodal cues provide redundant information for bumblebees when the stimulus is visually salient, but facilitate red target detection in a naturalistic background. *PLoS One* 12, e0184760. doi: 10.1371/journal.pone.0184760

Thomson, J. D., and Wilson, P. (2008). Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *Int. J. Plant Sci.* 169, 23–38. doi: 10.1086/523361

Wedzony, M., and Filek, M. (1998). Changes of electric potential in pistils of petunia hybrida hort. and brassica napus l. during pollination. *Acta Physiol. Plant.* 20, 291–297. doi: 10.1007/s11738-998-0061-x

Willmer, P. (2011). *Pollination and floral ecology* (Princeton: Princeton University Press).

Wilson, P., Castellanos, M. C., Wolfe, A. D., and Thomson, J. D. (2006). Shifts between bee and bird pollination in penstemons. *Plant–pollinator Interact.: Specialization to Generalization.* 47–68.

Wolf, L. L., Stiles, F. G., and Hainsworth, F. R. (1976). Ecological organization of a tropical, highland hummingbird community. *J. Anim. Ecol.* 45, 349–379. doi: 10.2307/3879

Zanne, A. E., Tank, D. C., Cornwell, W. K., Eastman, J. M., Smith, S. A., FitzJohn, R. G., et al. (2014). Three keys to the radiation of angiosperms into freezing environments. *Nature* 506, 89–92. doi: 10.1038/nature12872

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All the Colors of the Rainbow: Diversification of Flower Color and Intraspecific Color Variation in the Genus *Iris*

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Floral color plays a key role as visual signaling and is therefore of great importance in shaping plant-pollinator interactions. *Iris* (*Iridaceae*), a genus comprising over 300 species and named after the Greek goddess of the colorful rainbow, is famous for its dazzling palette of flower colors and patterns, which vary considerably both within and among species. Despite the large variation of flower color in *Iris*, little is known about the phylogenetic and ecological contexts of floral color. Here, we seek to resolve the evolution of flower color in the genus *Iris* in a macroevolutionary framework. We used a phylogenetic analysis to reconstruct the ancestral state of flower color and other pollination-related traits (e.g., the presence of nectar and mating system), and also tracked the evolution of color variation. We further explored weather floral trait transitions are better explained by environmental or pollinator-mediated selection. Our study revealed that the most recent common ancestor likely had monomorphic, purple flowers, with a crest and a spot on the fall. The flowers were likely insect-pollinated, nectar-rewarding, and self-compatible. The diversity of floral traits we see in modern irises, likely represents a trade-off between conflicting selection pressures. Whether shifts in these flower traits result from abiotic or biotic selective agents or are maintained by neutral processes without any selection remains an open question. Our analysis serves as a starting point for future work exploring the genetic and physiological mechanisms controlling flower coloration in the most color-diverse genus *Iris*.

Keywords: pollination syndrome, flower color evolution, color variation, mating system, pollinator shifts, shelter reward, nectar reward, ancestral trait reconstruction

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INTRODUCTION

Visual floral traits, flower color in particular, are important features that shape plant interactions with the surrounding environment (Schaefer and Ruxton, 2011; Willmer, 2017). Flowers show enormous variation in color among closely related taxa, and even between or within natural populations of the same species (i.e., flower color polymorphism) (Gigord et al., 2001; Warren and Mackenzie, 2001; Narbona et al., 2018; Souto-Vilarós et al., 2018). The factors that drive flower color evolution in highly diverse genera or families, particularly within a species, remain an open area of research.

Flower color is one of the most important characters for signaling to animal pollinators (Schiestl and Johnson, 2013). Therefore, pollinators are often perceived as one of the primary selective agents influencing flower color. Differences in the visual capabilities of pollinators can impose variable selective pressure on flower color, leading to variation (Wester and Lunau, 2017; de Camargo et al., 2019). In some plant species color transitions represent an adaptation to different, sometimes new, suites of pollinators (Armbruster et al., 2000; Fenster et al., 2004). For instance, in some plant genera, color is the best predictor of a transition between insect and bird pollination (Sutherland and Vickery, 1993; Roguz et al., 2018).

While flower color is a central visual cue to animal pollinators, the floral reward also plays a key role in shaping the interaction (Roy et al., 2017; Parachnowitsch et al., 2018; Roguz et al., 2018, 2019). Typically, plants provide a food reward of either pollen or nectar. Of these, nectar is perhaps the most important in an evolutionary sense (Simpson and Neff, 1983; Canto et al., 2011). Although it is a strong attractant, producing nectar is physiologically costly, and thus in several cases, the ability of flowers to produce nectar has been lost (Little, 1983; Dafni, 1984; Sletvold et al., 2016). In some plant families, e.g., Orchidaceae, the presence or absence of a nectar reward may be correlated with flower color. Non-rewarding, sexually deceptive orchids often have brightly colored flowers (Spaethe et al., 2010), while species with nectar often have green or white colored flowers (Duffy and Stout, 2011). The lack of a food reward might result in, or be the result of, the development of new rewarding characters that attract potential pollinators. These new attractants may include changes in flower color and size (Gigord et al., 2001; Vereecken and Schiestl, 2009). For example, night-sheltering reward systems without a food reward are often associated with large, dark flowers (Dafni et al., 1981; Sapir et al., 2005, 2006; Watts et al., 2013; Lavi and Sapir, 2015). Dark petals with their highly absorptive surfaces may result in higher temperatures inside the flower, which may benefit flower visitors (Sapir et al., 2006; Watts et al., 2013). As a result, in such systems flower size and color, but not food rewards, are typically under strong selection (Vereecken et al., 2013; Lavi and Sapir, 2015; Pellegrino et al., 2017).

Flower color changes might also be associated with a shift in plant mating system (Landis et al., 2018). The majority of flowering plants have both male and female reproductive parts, however, the mutualistic interactions with pollinators enable most of these plants to outcross (Goldberg et al., 2010). Shifts in flower color and pollination vectors may result in changes in mating system (i.e., selfing or outcrossing). Some species that rely on pollinators have lost the ability to self-fertilize, becoming self-incompatible, while in other cases the reverse occurred (Landis et al., 2018). Strong selection on floral traits, including color, is expected in self-incompatible taxa relying completely on animal-mediated pollination. Whereas in self-compatible taxa, this animal-mediated selection is expected to weaken since pollinator visitation may no longer be required for reproduction (Anderson and Busch, 2006). Thus, while pollinators often play a critical role in the evolution of flower color, this may not always be the case.

Plants experience a myriad of interactions with animals, both mutualistic and antagonistic. The strength and direction of selection that these agents exert on flower color may differ (Irwin et al., 2003). For example, flower color may have evolved as an adaptation against fungi and/or herbivores (Chalker-Scott, 1999; Chittka and Schürkens, 2001). Irwin et al. (2003) found that herbivores and pollinators exert opposing selection on flower color in *Raphanus sativus*. In this system, because both herbivores and pollinators prefer lighter flowers, dark flowers persist, thus maintaining a stable color polymorphism.

In some taxa, however, there is little or no association between color and interacting animals (Armbruster, 1996). Instead, variation in pigmentation within and among closely related taxa may be maintained by selection related to environmental heterogeneity (Warren and Mackenzie, 2001). The three main groups of pigments responsible for color in plants, flavonoids, carotenoids and betalains, play a functional role in plant physiology (Schaefer and Ruxton, 2011). Flavonoids, for example, are known to function as a response to plant stress caused by drought (Warren and Mackenzie, 2001), cold (Chalker-Scott, 1999; Harborne and Williams, 2000), and nitrogen deficiency (Bonguebartelsman and Phillips, 1995). They also protect plants against damage caused by UV radiation or visible light (Chalker-Scott, 1999; Tanaka et al., 2008). Flower color mediates plant interactions with both the abiotic and biotic environment, and thus exploring the drivers that lead to flower color diversity is of key importance to understand the evolution of plant diversity.

Transitions in flower color are common across the phylogenies of Angiosperm lineages (Streisfeld and Rausher, 2011; Wessinger and Rausher, 2012, 2014; Martins et al., 2016; Roguz et al., 2018). The diversity of flower color among and within these modern lineages suggests that most of these transitions must have been adaptive (Rausher, 2008). While the mutations causing flower color shifts are well understood at the biochemical level (Grotewold, 2006), the broader macro-evolutionary drivers of flower color diversity have only been studied in few plant groups (Landis et al., 2018; Ng and Smith, 2018). To understand these macro-evolutionary forces, we need to explore plant lineages exhibiting a diversity of flower colors.

The genus *Iris* is one of the most diversified genera in Asparagales. *Iris* comprises over 300 species (Royal Botanic and Gardens, 2020), which are widespread throughout the Northern Hemisphere (Goldblatt and Manning, 2008), with the greatest number of endemics occurring in the Mediterranean and Asia (Wilson, 2006). Although some irises are found in mesic or even wetland environments, most species grow in desert, semi-desert, or dry, rocky, montane habitats (Wilson, 2006). Members of this genus display a remarkable variety of flower colors, ranging from extremely dark, purple flowers, through violet and pink, to yellow and white flowers (Mathew, 1989; Sapir and Shmida, 2002; Sapir et al., 2002). This dazzling palette of colors and patterns seen in *Iris* flowers may be associated with their wide variety of life histories, pollination and mating systems, and habitats. Thus irises represent an outstanding model to study evolutionary biology and speciation in plants, especially in the context of flower color (Crespo et al., 2015).

To understand the unusual flower colors and color patterns in irises we investigated the evolution of flower color and several related traits, including pollinator type, nectar reward, and mating system, across the entire phylogeny and geographic range of the genus. By determining the ancestral state of *Iris* traits and comparing it to the traits of modern species, we should be able to shed light on which traits are the causes and which are the effects. To this end, we asked the following specific questions, first, what was the ancestral state of flower color and related traits in *Iris*, and second, are floral trait transitions from ancestral to modern states better explained by environmental or pollinator mediated selection?

MATERIALS AND METHODS

Phylogenetic Tree

To set up our analyses on the evolution of flower color in irises, we first created a phylogenetic tree for the genera, based on a database of sequences. We created the database using six sequences, five plastid genomes (*matK*, *trnL*, *trnK*, *NADPH,* and *rbcL*) and one nuclear internal transcribed spacer (*ITS*). All of the gene sequences were acquired from GenBank (Accession numbers in **Supplementary Material 1**) and downloaded using the MatPhylobi program (Kranas et al., 2018), which is a command-line tool for constructing taxonomic data sets for phylogenetic inference based on NCBI data. The sequences downloaded represent 227 *Iris* taxa which include 215 species, 10 subspecies and two varieties (see full list and ranks in **Supplementary Material 1**). Our analysis included all taxa with data available in GenBank. This taxa sampling covers the floral and geographic diversity of the genus. To create the phylogenetic database in MatPhylobi, we seeded *Iris pumila* as the representative *Iris* species and selected *Crocus vernus*, *Morea inclinata,* and *Dietes robinsoniana* as outgroups, based on their previously established sister relationships (Goldblatt et al., 2002; Wilson, 2006). Overall, taxon sampling totalled 429 accessions and total gene coverage was approximately 53.7% (227 *Iris* taxa out of 431), with *matK* having the highest coverage (40.7%) and *ITS* the lowest coverage (9.95%).

To refine the database, all sequences were independently aligned using the multiple alignment program MAFFT (version 7; Kuraku et al., 2013; Katoh et al., 2017) (method = "localpair," incorporating local pairwise alignment information, maxiterate = 1,000). Subsequently, we imported all of the alignments into Mesquite for visual inspection (version 3.6; Maddison and Maddison, 2018). Poorly aligned positions and divergent regions were eliminated using the Gblocks program (Version 0.91b, Castresana, 2000; Talavera and Castresana, 2007). The trimmed alignments were then concatenated with catfasta2phyml into a single aggregate alignment[1]. For each sequence, we selected the appropriate evolutionary model based on its specific characteristics using ModelTest-NG (version 0.1.3; Darriba et al., 2020; **Supplementary Material 2**).

Using our refined database of sequences, we used RAxML to generate our final phylogenetic tree. RAxML uses a series of maximum-likelihood (ML) tests to generate the tree (version 8.0; Stamatakis, 2014). To find the best phylogenetic tree, we used a bootstrap analysis with 1,000 replicates. Only bootstrap values (i.e., the probability that respective groups of taxa are present in the true phylogeny) higher than 50% were presented on the tree.

Floral and Habitat Characters

Once we had our phylogenetic tree, we prepared a database describing the diversity of irises, including 16 traits related to flower color, reproduction, habitat and distribution (**Table 1**). A majority of these traits were determined using Mathew (1989), which is a compilation of information about irises around the world. Additional sources included regional floras, the scientific literature, as well as internet sources[2,3]. Table with all data available in **Supplementary Table 1**.

Iris flowers have six sepals, which are usually divided into two types: three falls that droop downwards and three standards that are upright. These two sepal-types often have distinct characteristics (**Figure 1**).

We assessed flower color and eight color-related traits. Because of the wide range of taxa and variation in literature sources regarding those taxa, our first color metric was a simple assessment of flower tepals color based on human perception. Although UV reflecting flower parts may play an important role in communication with pollinators, we were not able to include this information in our study due to a lack of relevant data. The availability of flower reflectance data of any sort is limited among *Iris* taxa and do not allow genus-level analysis. For each taxon, the color was assessed and categorized into seven flower color categories: maroon, orange, pink, purple, red, yellow and white. In irises the differences between blue, violet and purple flowers are vague, therefore we coded species with flowers in these colors as purple. Polymorphic taxa fell into several categories (e.g., *I. lutescens* with blue and yellow morphs). Taxa with bi-colored flowers (e.g., *I. narbuttii* with yellow standards and violet falls) were coded as representing two categories. Second, we assessed flower pigment. Taxa were categorized as having anthocyanins (pink, purple, or red flowers) or carotenoids (orange and yellow flowers) as the major pigment, or lacking anthocyanins flavonoids and carotenoids (white or creamy specimens). As in the previous flower color trait, polymorphic and bi-colored flowers were coded into multiple categories. Several previous studies have used these pigment categories, which allow comparisons among flower taxa while eliminating differences related to color perception or habitat influence (Arista et al., 2013; Smith, 2015; Ellis and Field, 2016; Landis et al., 2018).

Flower color polymorphic species with white-flowered morphs (i.e., lacking anthocyanins and carotenoids) were assigned based on the colored morph (with pigment) to a pigment trait category as has been done in previous studies (Smith, 2015; Ellis and Field, 2016; Landis et al., 2018). Therefore, to

[1]https://github.com/nylander/catfasta2phyml

[2]http://www.signa.org

[3]http://www.wiki.irises.org

TABLE 1 | Results of phylogenetic signal analysis.

Trait	Trait type	Phylogenetic signal	
Bi-colored flowers	Categorical binnary	Estimated D:0.52	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.001
Color polymorphism	Categorical binnary	Estimated D:0.70	Probability of $E(D_r)$:0.002 Probability of $E(D_B)$:0
Continuous flower color variation	Categorical binnary	Estimated D:0.89	Probability of $E(D_r)$:0.114 Probability of $E(D_B)$: 0
Beard on the falls	Categorical binnary	Estimated D:-0.36	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.99
Crest on the falls	Categorical binnary	Estimated D:-0.30	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.95
Color spot on the falls	Categorical binnary	Estimated D:0.75	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0
Diameter	Continuous	Phylogenetic signal K : 0.0022662 Phylogenetic signal lambda : 0.616142	*P*-value: 0.159 *P*-value: 0.000
Mating system	Categorical binnary	Estimated D:-0.14	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.001
Nectar production	Categorical binnary	Estimated D:	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.001
White morphs	Categorical binnary	Estimated D:0.66	Probability of $E(D_r)$:0 Probability of $E(D_B)$:0.95

For all of the binary traits (all but diameter) the signal is expressed as a D-value (probability of $E(D_r)$: a p value, giving the result of testing whether D is significantly different from one, probability of $E(D_B)$: a p value, giving the result of testing whether D is significantly different from zero; caper package, "phylo.d" function). For the continuous trait (diameter) the strength of the phylogenetic signal is expressed as the Blomgergs's K and Pagels λ (phytools package in R, version 0.5–20; Revell, 2012).

capture the presence of white morphs, we added a white/non-white binary trait category. We also had binary trait categories for bi-colored/not bi-colored flowers, poly/monomorphic flowers (not including white morphs), and continuous/non-continuous flower colors (e.g., where color morphs occur across a continuous color gradient instead of discrete color morphs, as is the case in *I. petrana*). All species with continuous flower colors were also described as having polymorphic flowers. Finally, we also assessed the presence or absence of three traits that contribute to the overall visual display of *Iris* flowers: beard, crest, and spot. In the centre of the fall, irises either have a hairy or bristly tuft called a beard, or a cockscomb-like crest. Additionally, many *Iris* taxa have a signal patch (*hereafter* spot) of a different or stronger color on the fall (Mathew, 1989).

We also assessed several traits related to flower attraction and reproduction that potentially played a role in the evolution of *Iris* flowers: corolla diameter, pollinator type, mating system (self-compatibility vs. self-incompatibility), and presence of nectar. Apart from diameter, the rest of these data were ascertained either from the published literature or from personal communication (**Supplementary Table 1**). In most cases, pollinator type was not described to taxon level, therefore to be conservative we included only two broad groups, insects or birds.

Finally, for all studied taxa with available information, we assessed habitat type, elevation (maximum height), and geographical range. There were 10 habitat categories, with some taxa falling into several categories (e.g., desert and stony slope). Irises are found across the entire Northern Hemisphere and we identified the specific regions in which each taxon can be found. To assess whether there is any pattern relating flower color to geographical range, we used the geographic data to overlay the proportion of taxa with different flower color categories onto a QGIS map using QGIS 3.10.5 (Szczepanek, 2012; QGIS Development Team, 2020). For this visualization, each pie chart is located in the center of the specific geographical region, as calculated by QGIS.

Overall, we have flower color data for all 227 taxa. Coverage of other traits is less complete: corolla diameter 92%, pollinator type 56%, mating system 51%, and nectar availability 59%. We were able to capture data about habitat type and geographical range for all taxa, but only found elevation data for 90% of taxa.

It is important to mention that our study is biased by the sources and quality of the data we were able to obtain to populate our trait table. Ideally, we would have liked to have more resources and resolution around some of these traits, particularly around the reproductive systems of the studied irises. Also, although flower scent and color may be derived from the same biosynthesis pathways (Delle-Vedove et al., 2017) and scent plays an important role in pollinators attraction (Dormont et al., 2019) we did not include scent in our analysis due to a general lack of data regarding iris floral volatiles. That said, we are confident that we captured what data is available for these taxa and have made our inferences on that basis.

Ancestral State Reconstruction and Phylogenetic Signal

We used our phylogenetic tree and trait databases to determine the ancestral state of flower color and related traits in *Iris*. The ancestral states were inferred from ultrametric tree, generated using the *chronos* function in "ape" package (with the age of the tree set to one, value of smoothing parameter lambda = 0 based on log-likelihoods; Paradis et al., 2004). For each trait, we first determined the appropriate transition probability model, choosing among ER—equal rate, SYM—symmetrical rate, and ARD—all-rates different, using a log likelihood ratio analysis. In all cases, the ARD transition probability model was chosen because it had the highest likelihood value. However, in our analyses testing the ancestral states of bi-color, continuous, and polymorphic flowers our results suggested that the ARD model was overfitted (number of detected trait changes with the ARD model was several million vs. a thousand in the ER model), therefore for these analyses we used the ER model instead. To compute the total number of character changes between all states of binary trait categories, we used the make.simmap function in the phytools package (100 simulations across the tree, Revell, 2012).



FIGURE 1 | Flowers of selected *Iris* species [**(A)** *I. atropurpurea*, **(B)** *I. bismarckiana,* **(C)** *I. fulva,* **(D)** *I. historio,* **(E)** *I. loretti*, **(F)** *I. lutescens*, **(G)** *I. mesopotamica*, **(H)** *I. petrana,* **(I)** *I. pumila*, **(J)** *I. reticulata*, **(K)** *I. setosa* **(L)** *I. virginica*].

For polymorphic traits (i.e., flower color and pigment), we inferred the ancestral character states with the R package *corHMM* (Beaulieu et al., 2013) using the "rayDISC" function, which specifically accommodates polymorphic characters. For all of the binary trait categories, we estimated the ancestral character states using a continuous-time Markov chain model (Mk model,

phytools package). For the ancestral state reconstruction of the continuous trait, diameter, we used the FastAnc function in the phytools package (version 0.5–20; Revell, 2012).

Testing the strength of the phylogenetic signal reveals a tendency for related taxa to resemble each other more than taxa drawn at random from the same tree. For all of the binary trait we measured a D-value (Fritz and Purvis, 2010), which is a measure of phylogenetic signal dedicated to this kind of dataset (caper package, "phylo.d" function, Orme et al., 2013). The strength of the phylogenetic signal on continuous data was calculated as the Blomgergs's K (Blomberg et al., 2003) and Pagels λ (Pagel, 1999) (phytools package in R, version 0.5–20; Revell, 2012).

RESULTS

Phylogenetic Tree

Most of the described large-scale phylogenetic relationships found in previous studies were recaptured in our final tree (Wilson, 2004, 2009; Wilson et al., 2016; Jiang et al., 2018). That said, we decided to exclude *Iris darwasica* from the analysis, because of conflicting nesting results between our study and others. Specifically, we found that *I. darwasica* was assigned to the subgenus *Limniris,* but according to previous studies and floral characters this taxon belongs to the subgenus *Iris*, section *Regelia* (Khassanov and Rakhimova, 2012). Leaving such a wrongly assigned taxon in our tree may have biased all subsequent analyses.

There were some differences in the topology observed between the six sequence-specific trees and the final summarizing tree. For example, in two of the six trees that were rooted on *Crocus vernus* (*matK* and *ITS*), the outgroup *Dietes robinsoniana* resolved as nested within the *Iris* subgenus *Limniris* (low bootstrap; *hereafter* Bp).

As in previous studies, the topology of our final phylogenetic tree has two major subgenera (*Limniris* and *Iris*) and five minor subgenera (*Hermodactyloides, Nepalensis, Pardanthopsis, Scorpiris,* and *Xiphium*). All but *Limniris* were resolved as monophyletic. Within *Limniris*, there were two sections, *Limniris* (71 Bp) and *Lophiris*, with the first section containing the majority of the taxa belonging to the genus. Within the subgenus *Iris,* there were six sections: *Pardanthopsis* (99 Bp), *Psammiris* (56 Bp), *Pseudoregelia* (99 Bp), *Oncocyclus* (86 Bp), *Regelia* (87 Bp), *Hexapogon* (86 Bp), and *Pogon* (**Figure 2**).

A full discussion of the final tree and contributing sub-trees is presented in **Supplementary Material 3**.

Floral and Habitat Characters

Of the 226 *Iris* taxa included in this analysis, 49.3% had flowers in shades of purple and 24.5% had yellow flowers. The rest were distributed among maroon (14%), white (11%), pink (4%), orange (0.5%), red (0.5%). Most of the studied taxa were categorized as having anthocyanins (80%) and a third were characterized as having carotenoids (33%), with several taxa containing both pigments. Approximately 43% of the genus has white flower morphs or flower-parts (i.e., white standards or falls). There were 35 taxa categorized as having bi-colored flowers, 35 taxa categorized as having polymorphic flowers, and 23 taxa with continuous flower color variation. More than half of the *Iris* taxa have a crest (141 taxa), while over a third (85 taxa) have a beard; also, more than half of *Iris* taxa have a spot (143 taxa). *Iris* flowers range in diameter between 1.25 and 16.5 cm (MEAN $\pm$ SD: 6.2 $\pm$ 2.3 cm) (**Figure 3** and **Supplementary Table 1**).

The majority of *Iris* taxa are pollinated by insects, primarily bees (members of *Andrena, Anthophora, Apis, Colletes, Emphoropsis, Eucera, Hylaeus, Lasioglossum, Nombus, Osmia, Tetralonia,* and *Xylocopa*). Several *Iris* taxa, however, are also pollinated by flies (e.g., *I. bracteata, I. gracilipes, I. palaestina*) and butterflies (e.g., *I. fulva*). Hummingbird pollination was observed in four *Iris* taxa: *I. cristata, I. fulva, I. hexagona,* and *I. missouriensis*. Most *Iris* taxa produce nectar and are self-compatible. The majority of taxa that do not produce nectar are also self-incompatible, have a beard, and belong to the *Oncocyclus* and *Regalia* sections.

Taxa with purple and yellow flowers are almost equally distributed on all continents, although yellow morphs are rare in South Asia. Polymorphic and bi-colored taxa are more prevalent in the Middle East than in other regions, and bi-colored purple-white species are completely absent from North America (**Supplementary Figure 1**).

Ancestral States of Floral Traits, Their Transitions, and Phylogenetic Signal

The ancestral flower color of *Iris* was most probably purple (**Figure 4**) and anthocyanin-based (**Figure 5**), without the ability to produce white flower morphs (**Supplementary Figure 2**). Most internal nodes also exhibited purple flowers with anthocyanin pigments (**Figures 4**, **5**). Having color-monomorphic flowers, where all parts of the flower are the same color, was likely the ancestral state among *Iris* and remained so in the early nodes. Taxa with polymorphic, continuous, or bi-colored flowers all seem to be derived states that have arisen and been lost several times (**Supplementary Figure 3**). The most recent common ancestor of *Iris* likely had flowers with a crest and a spot (**Supplementary Figure 4**), and was self-compatible, insect-pollinated, and nectar-rewarding (**Table 2A** and **Figure 6**).

Flower color and pigment are variable, with most of the color and pigment transitions being from yellow/carotenoid-dominated flowers to purple/anthocyanin-dominated flowers. The transitions between purple and white were also common. Of the taxa involved in these purple-white shifts, a majority represent bi-colored flowers, where either the fall or standard is white (**Table 2B**). The ability to produce white flower morphs is widespread across the phylogenetic tree (1,098 transitions on average; *hereafter*—average value based on 100 simulations), but labile, with several changes back and forth. In contrast, having monomorphic flowers with a crest persisted for most of the time. The small number of transitions between color monomorphic or polymorphic, and between having a crest or a beard suggests that these traits tend to be conserved. The proportion of time that *Iris* lineages have had a beard is comparatively short and this trait probably evolved once in a common ancestor of the

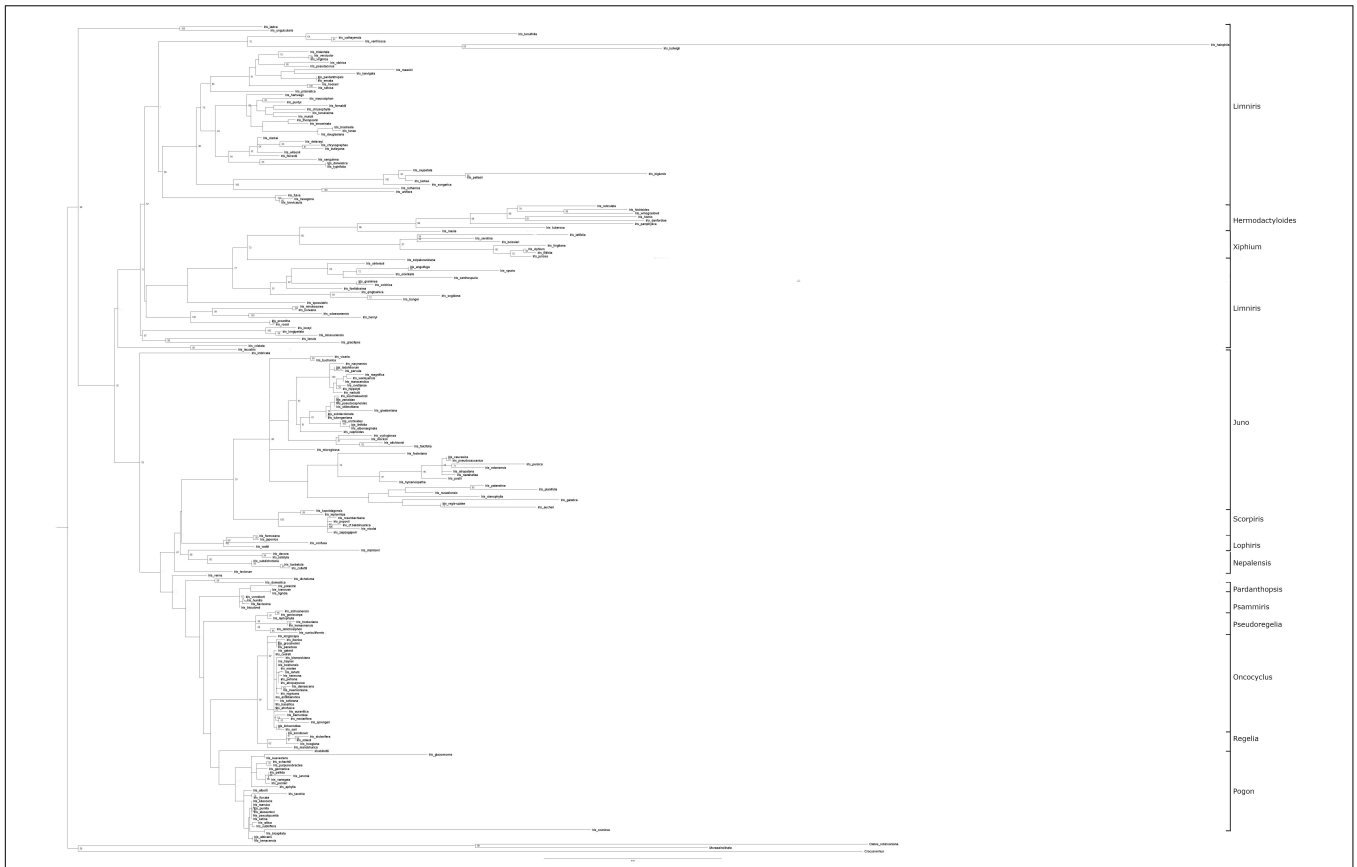


FIGURE 2 | Maximum likelihood tree inferred from analysis of combined five plastid genomes (*matK*, *trnL*, *trnK*, *NADPH* and *rbcL*) and one nuclear internal transcribed spacer (*ITS*) sequences. The bootstrap values are given along the branches (only values > 50 presented).

subgenera *Pogon, Oncocyclus, Pseudoregelia, Regelia, Psammiris,* and *Pardanthopsis,* and two times more recently for *I. falcifolia* and *I. imbricate*. Unlike the crest and the beard, the presence of a spot varied more across taxa, with several changes among closely related taxa (**Table 2A**).

The ability to self-pollinate and produce nectar was conserved for most of the time (**Table 2A**), and only lost once in the common ancestor of the subgenera *Pogon* and *Oncocyclus*. Both self-compatibility and the ability to produce nectar were regained several times. Even though an overwhelming majority of taxa are, and always have been, insect-pollinated, we found two independent shifts toward bird pollination (**Figure 6**), as well as several reversals from birds to insects (**Table 2A**).

Across the *Iris* lineage, we found all the studied binary traits to be phylogenetically conserved. Among them, the distribution of the states for the half of the traits (presence of bi-colored flowers, color polymorphism, continuous flower color and color spot on the falls) proofed to be distributed as expected under the Brownian motion model of evolution ($0<D<1$). For the second half (presence of beard, crest, nectar, or mating system) the distribution of the states was more phylogenetically conserved than the Brownian expectation ($D<0$). We also found a relatively strong phylogenetic signal for the flower diameter, however the significant value was obtained only for Pagel's λ (**Table 1**).

DISCUSSION

Iris flowers display a wide diversity of colors and color patterns among and within species. The diversity seen in modern irises is the result of several changes over evolutionary history in the genus. In this study, we reconstructed the traits of the most recent common ancestor of the *Iris* genus. This ancestor likely had monomorphic, one-colored purple flowers, with a crest and a spot on the falls. The flowers were likely insect-pollinated, nectar-rewarding, and self-compatible. Since then, the genus has diverged to include over 300 taxa that exhibit a wide range of colors and patterns, including polymorphic, continuous, and bi-colored flowers. Additionally, some taxa now have a beard, instead of a crest, have lost the spot (and sometimes regained it), and some are now self-incompatible and rewardless. There have been a few shifts from insect-pollination to bird-pollination, and occasionally back again. Thus, by comparing these derived states to the ancestral state, and placing them in the context of the *Iris* distribution and reproductive system, we can infer some of the drivers of floral color diversity in *Iris*.

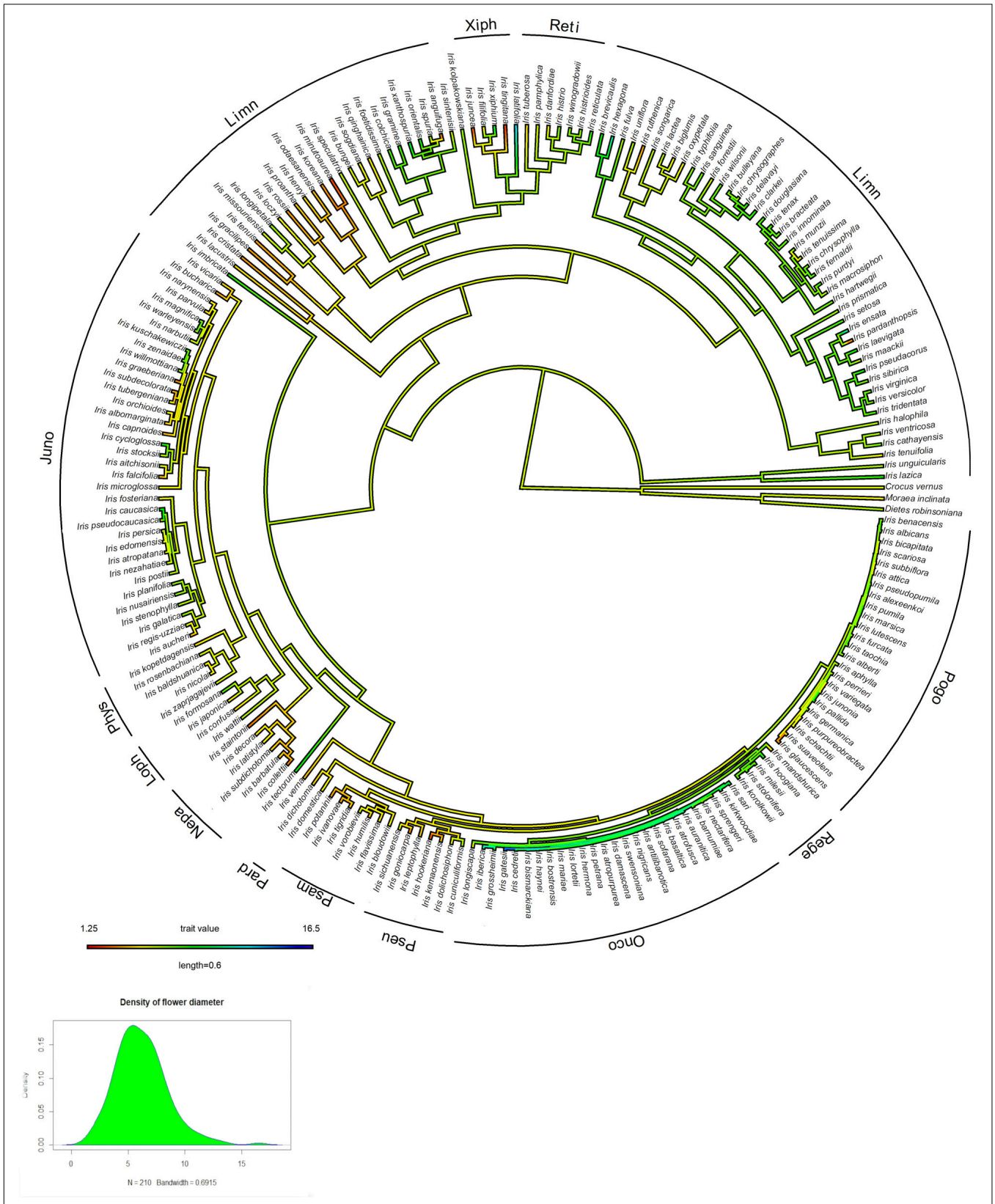


FIGURE 3 | Maximum-likelihood ancestral state reconstruction of corolla diameter [A] and histogram showing representation of the distribution of the variables [B].

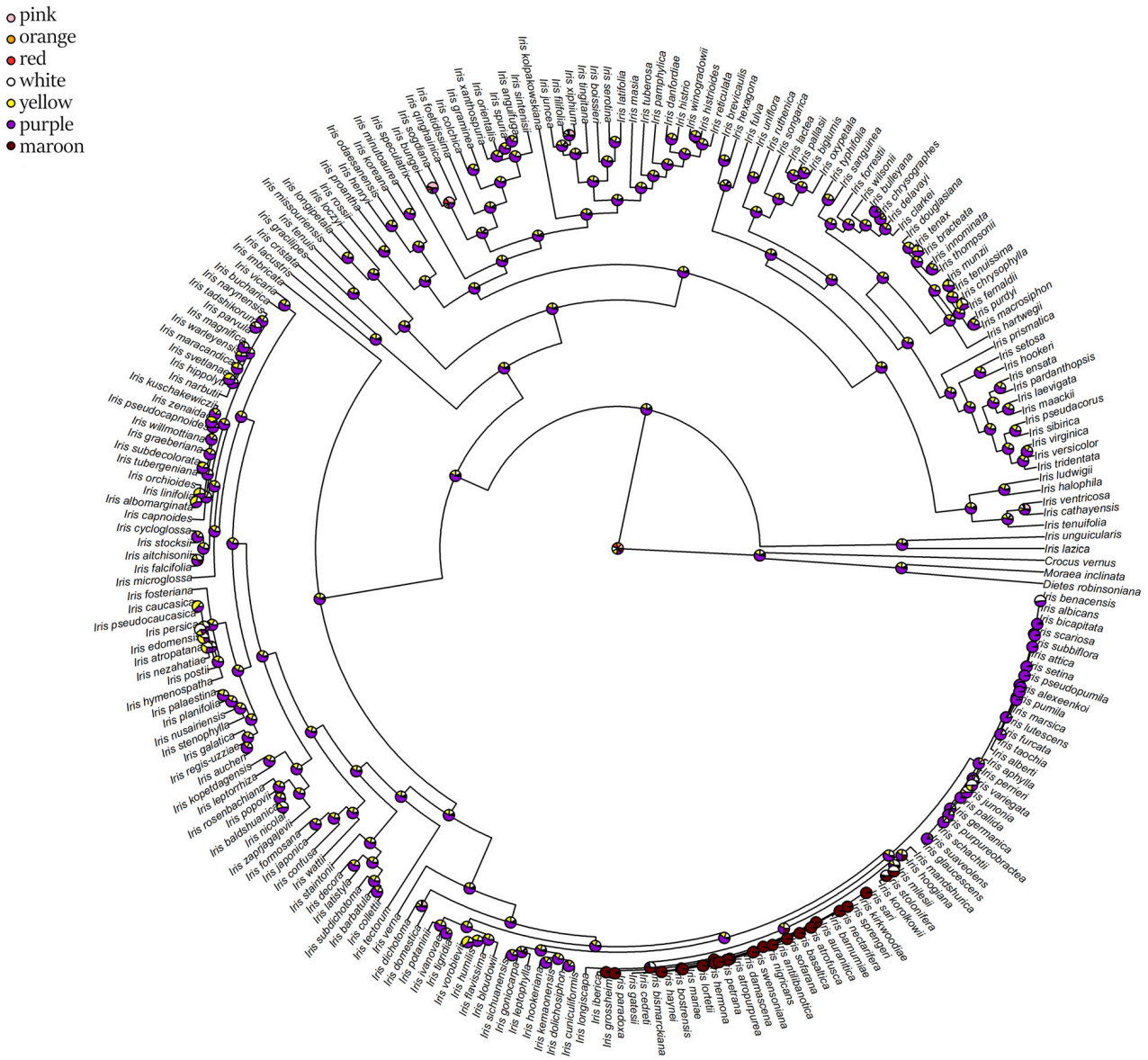


FIGURE 4 | Estimation of ancestral states of flower colors among studied *Iris* taxa calculated using maximum likelihood across the posterior distribution.

Flower color is the most variable trait of those we studied in the genus *Iris*. Some of that diversity is likely the result of mutations, which can cause up- or down-regulation of specific genes, leading to differences in the amount of synthesized pigment and in color shades (Durbin et al., 2003). The correlation between flower color diversity and anthocyanin synthesis is common not just in *Iris*, but across many genera, e.g., *Petunia* and *Ipomea* (Durbin et al., 2003). Unlike some of the other groups, however, the flower color diversity in *Iris* is not just related to the presence or absence of anthocyanin pigments, but also the result of variation in colors produced by anthocyanins (Warren and Mackenzie, 2001). Moreover, in *Iris* we also observed many cases of bi-colored flowers, which may be the result of tissue-specific variation in anthocyanin production, or the loss of anthocyanins in the standard or the fall.

Color polymorphism, continuous coloration, and bi-colored flowers arose multiple times across the *Iris* phylogeny, and many are maintained to this day (Wang et al., 2016). The maintenance of color polymorphism is not common in other genera. Typically, one of the color morphs will eventually be lost and the taxa will again be monomorphic (Ellis and Field, 2016). The maintenance of stable color polymorphism, and other color variations, in *Iris* may be caused by an absence of selective disadvantage on color in these taxa or by stabilizing selection exerted by multiple agents (reviewed in Sapir et al., in review). For example most populations of *I. lutescens* and *I. pumila* are polymorphic and neutrally distributed in space (Wang et al., 2016), but in some

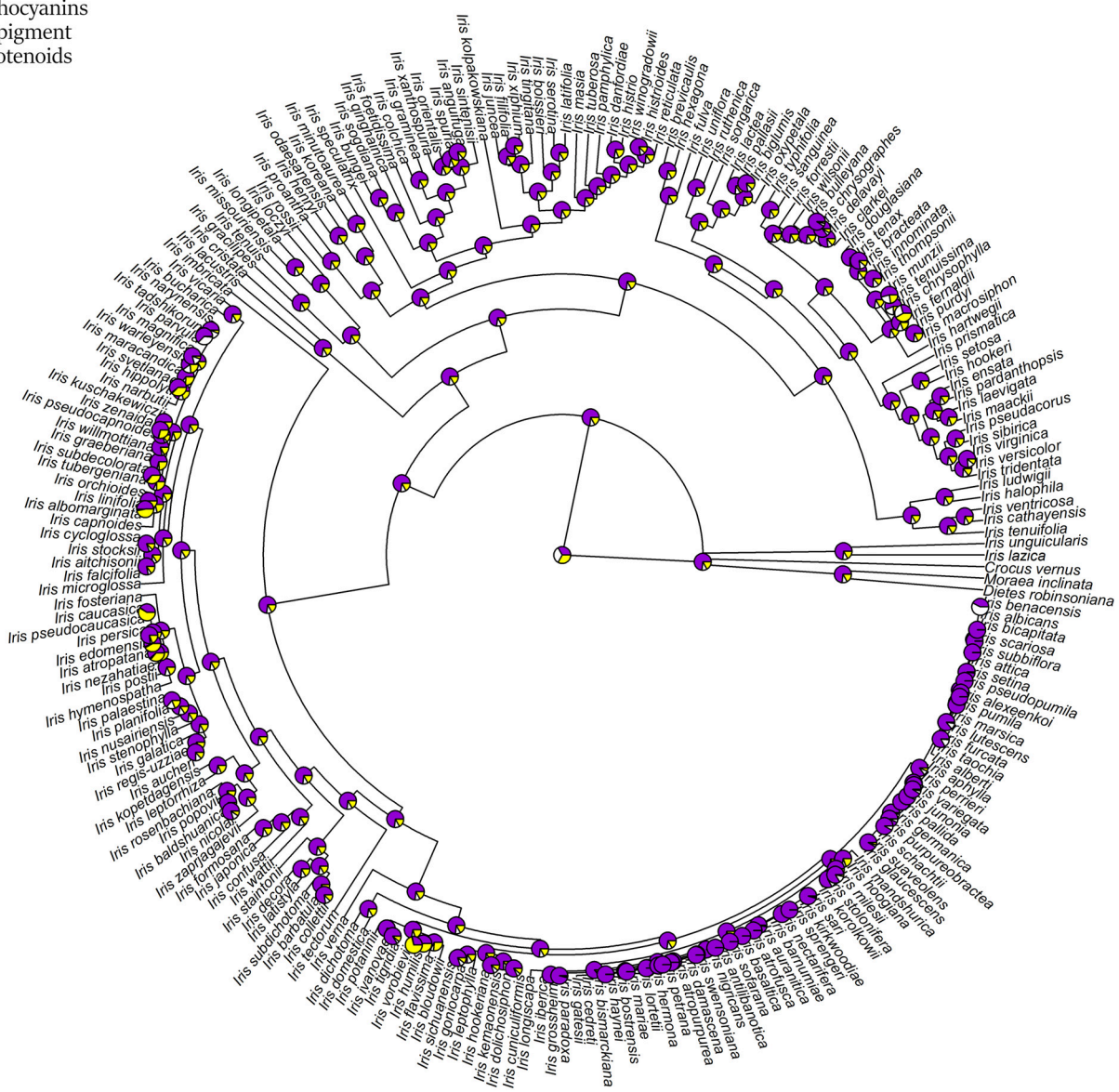


FIGURE 5 | Estimation of ancestral states of flower pigments among studied *Iris* taxa calculated using maximum likelihood across the posterior distribution. White and creamy flowers were coded as lacking anthocyanins flavonoids and carotenoids and are represented as white; maroon, purple, pink, or red flowers were coded as having anthocyanins and are represented in purple; yellow or orange flowers were coded as having carotenoids and are represented in yellow.

populations color variation may be due to divergence (Souto-Vilarós et al., 2018). Several factors may maintain this neutral distribution, including environmental heterogeneity (Tucić et al., 1998). Additionally, while selection should lead to the fixation of one color morph, polymorphism may also be maintained over the long-term due to perennial life history and vegetative reproduction, which cause generation overlap in many *Iris* species (Gray and McKinnon, 2007).

Almost half of the studied *Iris* taxa produce white morphs, which is a common color-transition in many angiosperm taxa, resulting from shut-down of genes in the pigment biosynthesis pathway (Wessinger and Rausher, 2012). However, the modern *Iris* taxa with (only) white-colored flowers can be found in the subgenera *Scorpiris* and *Limniris*. For some *Iris* species white flowers may be an evolutionary dead-end, without possibility for reversal. Pigment loss may alter pollinator attractiveness (Waser and Price, 1981) or reduce the capacity to deal with environmental stress, such as drought (Ellis and Field, 2016), or a combination of the two (Warren and Mackenzie, 2001).

Like the high diversity of flower color and patterns seen across *Iris* taxa, corolla diameter seems to be fairly variable across the phylogeny. The visual attractiveness of flowers is substantially related to flower color and size. Although large flowers are costly (Teixido et al., 2016; Roddy, 2019), some of the species with large

TABLE 2A | Results of simulated stochastic character mapping on *Iris* phylogenetic tree (all-rates different selected as transition probability model).

	Beard/ No beard	Bi-colored (1) /no bi-colored (0)	Continuous color (1) /no continuous color (0)	Crest (1) /no crest (0)	Insect (1) /insect and bird (0)	Monomorphic (1) /polymorphic (0)	Nectar (1) /no nectar (0)	Self-compatible (1) /self-incompatible (0)	Spot (1) /no spot(0)	White forms (1) /no white forms (2)
Average number of changes between states Number of specific transitions	9.72	56.2	30.6	10.4	11.1	64.0	19.9	2.4	1,582	1,098
1 –> 0	5.05	24.3	8.54	6.26	5.17	38.5	6.32.	2.07	780	548
0 < – 1	4.67	32.0	22.1	4.16	5.96	25.5	13.6	0.33	802	550
Proportion of time spent in the state 1	0.09	0.13	0.08	0.83	0.91	0.83	0.97	0.95	0.51	0.56
Proportion of time spent in in the state 0	0.91	0.87	0.92	0.17	0.09	0.17	0.03	0.05	0.49	0.44

Number and time of transitions between studied Iris characters identified with the use of make.simmap function in the phytools R package. The estimations are based on 100 trees with a mapped character.

TABLE 2B | Matrix containing the maximum likelihood estimates of the transition rate of color and pigment on *Iris* phylogenetic tree (all-rates different selected as transition probability model).

	Pink	Orange	Red	White	Yellow	Purple	Maroon
Pink	NA	15	0.76	0.76	0.76	30.5	0.76
Orange	64.6	NA	23.2	0.76	0.76	0.77	0.76
Red	16.3	0.76	NA	0.76	0.76	0.76	0.76
White	0.76	0.76	0.76	NA	0.76	0.76	10.2
Yellow	0.76	0.76	0.76	0.76	NA	100	0.76
Purple	1.81	0.76	0.76	22.6	9.95	NA	0.76
Maroon	27.8	0.76	0.76	0.76	39.7	27.9	NA

	Ant	No & and car	Car
Ant	NA	13.9	22.9
No ant & car	100	NA	0.76
Car	100	0.76	NA

The maximum likelihood values identified with the use of rayDisc function in the corHMM R package. The estimations are based on 1,000 trees (pigment abbreviations: ant, anthocyanin-based; no & and car lacking anthocyanins flavonoids and carotenoids; car, carotenoids-based.

flowers in the genus *Iris* grow in desert or semi-arid habitats. This suggests that pollinator-mediated selection is more important in driving the evolution of large flowers (Lavi and Sapir, 2015; Souto-Vilarós et al., 2018). The strategy to produce big, showy, self-compatible flowers with lots of nectar is found in most *Iris* taxa and may increase mating success, especially when florivory is not a threat (Ghara et al., 2017). While positive pollinator-mediated directional selection on flower size may be a factor in some *Iris* taxa (Lavi and Sapir, 2015; Souto-Vilarós et al., 2018), this is not always the case (Ishii, 2005; Pellegrino, 2015).

Most *Iris* species are pollinated by insects, but in a few cases, shifts to bird pollination evolved. Several taxa of *Iris* have blue-violet or purple flowers, which tend to be associated with bee visitation because bees tend to have an innate preference for the blue range of wavelengths (Lunau and Maier, 1995; Dyer et al., 2015). Bees also have an innate preference for yellow flowers, and thus the common shift from purple to yellow flowers in *Iris* may be maintained by bee preferences (Giurfa et al., 1995; Lunau and Maier, 1995; Spaethe et al., 2001). This innate preference for both purple and yellow flowers may explain the equal seed set between color morphs in some polymorphic *Iris* populations (Imbert et al., 2014a,b; Souto-Vilarós et al., 2018).

Bird-pollinated flowers tend to be red or orange (Rodríguez-Gironés and Santamaría, 2004; Cronk and Ojeda, 2008), and these two colors have arisen several times in the *Iris* phylogeny. Red coloration may be a good predictor of bird-pollination in irises, but not vice-versa. There is only one taxon with red flowers, and it is bird-pollinated (*I. fulva*, Emms and Arnold, 2000; Martin et al., 2008), but other bird-pollinated species are not red (e.g., *I. missouriensis*, Lyon, 1973). This suggests that color is likely not the major trait driving pollinator-shifts from insects to birds in *Iris*. All the bird-pollinated taxa have wide, open and flat flowers that produce dilute nectar (Wesselingh and Arnold, 2000). Being able to access the flower (wide open, flat flowers) and obtain the preferred food reward (diluted nectar) seems likely

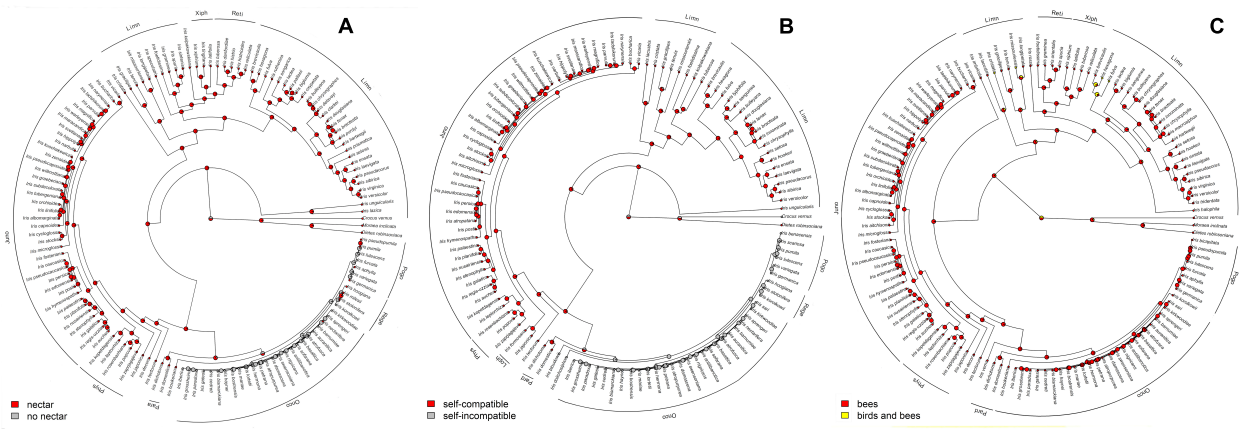


FIGURE 6 | Summarized stochastic mapping of mating system (self-compatibility vs. self-incompatibility), **(A)** presence or absence of nectar **(B)**, and pollinator type (insect vs. insect and bird) **(C)** in the genus *Iris* prepared using All Rates Different model with 1,000 iterations. Pie charts represent the proportion of the iterations showing either presence or absence of nectar at any given node.

to be the trigger for the transition to bird pollination. Shifting from purple to red flowers may be relatively easy because the anthocyanin biosynthesis pathway is relatively flexible (Warren and Mackenzie, 2001; Albert et al., 2011). Bird-driven transitions from purple to red flowers, however, would likely be countered by insect pollination because insects often prefer blue-violet (Lunau and Maier, 1995; Dyer et al., 2015) and yellow flowers (Giurfa et al., 1995; Lunau and Maier, 1995; Spaethe et al., 2001). This may explain why two out of the three extant bird-pollinated flowers are purple, and not red.

We found a strong signal for the association among floral structure, reward, and mating system. All taxa have either a crest or a beard, but never both. Although the ancestral *Iris* probably had a crest, the beard replaced the crest relatively early in the common ancestor of *Oncocyclus, Pogon,* and *Regalia*. Around the same time, this group of subgenera also lost the ability to produce nectar and became self-incompatible. Once the transition to a beard and self-incompatibility occurred in this section, it never changed back. In contrast, nectar production was regained several times. We also found, that white-purple and purple flower color, which are commonly found among members of these subgenera, are associated with self-incapability and a lack of nectar. While the association of these traits and their strong phylogenetic signal suggests the evolution of a monophyletic pollination syndrome, it is important to note the sampling of these traits was not similar across all species.

Nectar production requires regular and consistent water availability. In the dry regions inhabited by *Oncocyclus, Pogon,* and *Regalia* irises, water limitation could have been the agent of selection against nectar production, leading to the loss of this trait in their common ancestor. Once these irises became nectarless, the question becomes: how did they attract insect pollinators? Potentially, this is when the night-sheltering reward system, that is well-described in the *Oncocyclus* group, arose (Sapir et al., 2005; Monty et al., 2006; Vereecken et al., 2013; Watts et al., 2013). *Oncocyclus* irises are primarily pollinated by male *Eucera* bees (*Apidae, Eucerini*) that shelter in the flowers overnight (Sapir et al., 2005, 2006; Watts et al., 2013). Pollination occurs as the male bees visit multiple flowers before choosing the flower in which they will spend the night (Sapir et al., 2005; Monty et al., 2006). While it is possible that bees slept in the flowers before nectar was lost, after nectar-loss the sheltering component became the only reward. Thus, shelter may have become a replacement mechanism to attract pollinators.

The loss of nectar is associated with the transition from crest to beard. If a crest serves as a nectar guide, there will be no selection to maintain it in nectarless species. Instead, a beard might have selected by its advantage in alternative reward types, such as the derived sheltering-reward system. The role of the beard remains debatable, but it would be interesting to test whether the presence of a beard changes the airflow over the fall and impacts the rate of warming in these flowers, or whether it contributes for pollen deposition on stigma.

Another open question is why self-compatibility only arose in the closely related *Oncocyclus, Pogon,* and *Regalia* (except for *I. tenuis).* The loss of self-compatibility seems risky, but perhaps it was necessary to maintain genetic diversity. Most of the taxa in these subgenera occur in highly variable habitats in the Middle East and Western Asia. In a complex habitat mosaic, with patchy and extreme environmental conditions, being self-compatible may be maladaptive. Self-compatibility would naturally lead to selfing and inbreeding, thus reduce genetic diversity. Maintaining genetic diversity is particularly important for plants in highly variable and stressful conditions. It facilitates quick adaptation and increased population persistence, as a sort of insurance. Indeed, most of these taxa have highly variable flower traits, most notably in terms of color.

The nectarless *Oncocyclus, Pogon,* and *Regalia*, tend to have high levels of color polymorphism, continuous color variation, and bi-colored flowers. Within-species variation in flower color may be important in deceptive systems, since it impedes the learning ability of pollinators and leads to negative

frequency-dependent selection (Smithson and MacNair, 1997; Gigord et al., 2001; Imbert et al., 2014a). Another strategy to maintain the attractiveness of no-food rewarding species is flowering early in the season and attracting naïve pollinators (Imbert et al., 2014a). In any case, it is surprising that more taxa in these subgenera did not regain nectar production. Comparative studies have shown that nectarless taxa in general (Aragon and Ackerman, 2004; Sletvold et al., 2016), and *Iris* taxa specifically (Lavi and Sapir, 2015; Souto-Vilarós et al., 2018), are much more pollen limited than nectar producing taxa.

Irises cover a broad geographic range across a mosaic of habitat types, environmental stresses and pollinator types, which is likely the ultimate cause of the observed flower diversity. In general, some *Iris* taxa seem to have strong pollinator-mediated selection on floral traits, such as color and size. In other taxa, however, the environment seems to play a stronger selective role in one or more of these traits. The diversity of flower colors we see in *Iris*, likely represents a trade-off between conflicting selection pressures (Souto-Vilarós et al., 2018). Whether changes in flower color are the result of neutral processes without any selection, or whether these changes are tightly maintained by abiotic or biotic selective agents, remains an open question.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YS and KR conceived and designed the research. KR and MG performed analyses. KR, MG, and YS wrote the manuscript, with contributions from all authors. All authors collected the data.

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DEDICATION

This article is dedicated to Brian Mathew, leader in Iris taxonomy and ornamental bulb plants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.569811/full#supplementary-material

Supplementary Figure 1 | Map showing the total proportion of pigments among studied *Iris* taxa and the distribution of color morphs across the geographical range.

Supplementary Figure 2 | Summarized stochastic mapping of the presence of the white/no-white flowers in the genus *Iris* using All Rates Different model with 1,000 iterations. Pie charts represent the proportion of the iterations showing either presence or absence of the white/no-white flowers at any given node.

Supplementary Figure 3 | Summarized stochastic mapping of the presence of the poly/monomorphic **(A)**, continuous/non-continuous color **(B)**, bi-colored/not bi-colored **(C)** flowers in the genus *Iris* using All Rates Different model with 1,000 iterations. Pie charts represent the proportion of the iterations showing either presence or absence of the studied traits in flowers at any given node.

Supplementary Figure 4 | Summarized stochastic mapping of the crest **(A)**, beard **(B)**, or spot **(C)** presence/absence in the genus *Iris* using All Rates Different model with 1,000 iterations. Pie charts represent the proportion of the iterations showing either presence or absence of studied trait at any given node.

Supplementary Table 1 | Data table. Data include color and floral traits, habitat and source of data.

Supplementary Material 1 | Accession numbers of the gene sequences acquired from GenBank.

Supplementary Material 2 | Evolutionary models selected with the use of ModelTest-NG program.

Supplementary Material 3 | The contributing sub-trees of *Iris* genus; results and discussion of the phylogeny.

REFERENCES

Albert, N. W., Lewis, D. H., Zhang, H., Schwinn, K. E., Jameson, P. E., and Davies, K. M. (2011). Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. *Plant J.* 65, 771–784. doi: 10.1111/j.1365-313X.2010.04465.x

Anderson, I. A., and Busch, J. W. (2006). Relaxed pollinator-mediated selection weakens floral integration in self-compatible taxa of Leavenworthia (Brassicaceae). *Am. J. Bot.* 93, 860–867. doi: 10.3732/ajb.93.6.860

Aragon, S., and Ackerman, J. D. (2004). Does flower color variation matter in deception pollinated Psychilis monensis (Orchidaceae)? *Oecologia* 138, 405–413. doi: 10.1007/s00442-003-1443-9

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Armbruster, W. S. (1996). "Evolution of floral morphology and function: an integrative approach to adaptation, constraint, and compromise in *Dalechampia* (Euphorbiaceae)," in *Floral Biology*, eds D. G. Lloyd and S. C. H. Barrett (Boston, MA: Springer), 241–272. doi: 10.1007/978-1-4613-1165-2_9

Armbruster, S., Fenster, C., and Dudash, M. (2000). Pollination 'principles' revisited: specialization, pollination syndromes, and the evolution of flowers. *Scandanav. Assoc. Pollinat. Ecol.* 39, 179–200.

Beaulieu, J. M., O'Meara, B. C., and Donoghue, M. J. (2013). Identifying hidden rate changes in the evolution of a binary morphological character: the evolution of

plant habit in campanulid angiosperms. *Syst. Biol.* 62, 725–737. doi: 10.1093/sysbio/syt034

Blomberg, S. P., Garland, T., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1111/j.0014-3820.2003.tb00285.x

Bonguebartelsman, M., and Phillips, D. (1995). Nitrogen stress regulates gene-expression of enzymes in the flavonoid biosynthetic pathway of tomato. *Plant Physiol. Biochem.* 33, 539–546.

Canto, A., Herrera, C. M., García, I. M., Pérez, R., and Vaz, M. (2011). Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure. *Flora* 206, 668–675. doi: 10.1016/j.flora.2011.02.003

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mole. Biol. Evol.* 17, 540–552. doi: 10.1093/oxfordjournals.molbev.a026334

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol* 70, 1–9. doi: 10.1111/j.1751-1097.1999.tb01944.x

Chittka, L., and Schürkens, S. (2001). Successful invasion of a floral market. *Nature* 411, 653–653. doi: 10.1038/35079676

Crespo, M. B., Martínez-Azorín, M., and Mavrodiev, E. V. (2015). Can a rainbow consist of a single colour? A new comprehensive generic arrangement of the ' Iris sensu latissimo ' clade (Iridaceae), congruent with morphology and molecular data. *Phytotaxa* 232, 1–78. doi: 10.11646/phytotaxa.232.1.1

Cronk, Q., and Ojeda, I. (2008). Bird-pollinated flowers in an evolutionary and molecular context. *J. Exper. Bot.* 59, 715–727. doi: 10.1093/jxb/ern009

Dafni, A. (1984). Mimicry and deception in pollination. *Annu. Rev. Ecol. System.* 15, 259–278. doi: 10.1016/bs.abr.2016.10.005

Dafni, A., Ivri, Y., and Brantjes, B. M. (1981). Pollination of Serapias vomeracea Briq. *(Orchidaceae) by imitation of holes for sleeping solitary male bees (Hymenoptera). Acta Bot. Neerland.* 30, 69–73. doi: 10.1111/j.1438-8677.1981.tb00388.x

Darriba, D., Posada, D., Kozlov, A. M., Stamatakis, A., Morel, B., and Flouri, T. (2020). ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mole. Biol. Evol.* 37, 291–294. doi: 10.1101/612903

de Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Delle-Vedove, R., Schatz, B., and Dufay, M. (2017). Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Anna. Bot.* 120, 1–20. doi: 10.1093/aob/mcx055

Dormont, L., Joffard, N., and Schatz, B. (2019). Intraspecific variation in floral color and odor in orchids. *Int. J. Plant Sci.* 180, 1036–1058. doi: 10.1086/705589

Duffy, K. J., and Stout, J. C. (2011). Effects of conspecific and heterospecific floral density on the pollination of two related rewarding orchids. *Plant Ecol.* 212, 1397–1406. doi: 10.1007/s11258-011-99159911

Durbin, M. L., Lundy, K. E., Morrell, P. L., Torres-Martinez, C. L., and Clegg, M. T. (2003). Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. *Mole. Phylogenet. Evol.* 29, 507–518. doi: 10.1016/s1055-7903(03)00196-9

Dyer, A. G., Garcia, J. E., Shrestha, M., and Lunau, K. (2015). Seeing in colour: a hundred years of studies on bee vision since the work of the Nobel laureate Karl von Frisch. *Proc. Royal Soc. Vict.* 127, 66–72. doi: 10.1071/rs15006

Ellis, T. J., and Field, D. L. (2016). Repeated gains in yellow and anthocyanin pigmentation in flower colour transitions in the Antirrhineae. *Anna. Bot.* 117, 1133–1140. doi: 10.1093/aob/mcw043

Emms, S. K., and Arnold, M. L. (2000). Site-to-site differences in pollinator visitation patterns in a Louisiana Iris hybrid zone. *Oikos* 91, 568–578. doi: 10.1034/j.1600-0706.2000.910319.x

Felsenstein, J. (2012). A comparative method for both discrete and continuous characters using the threshold model. *Am. Natur.* 179, 145–156. doi: 10.1086/663681

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination Syndromes and Floral Specialization. *Annu. Rev. Ecol. Evol. System.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Fritz, S., and Purvis, A. (2010). Selectivity in Mammalian Extinction Risk and Threat Types: A New Measure of Phylogenetic Signal Strength in Binary Traits. *Conservat. Biol.* 24, 1042–1051. doi: 10.1111/j.1523-1739.2010.01455.x

Ghara, M., Ewerhardy, C., Yardeni, G., Matzliach, M., and Sapir, Y. (2017). Does floral herbivory reduce pollination-mediated fitness in shelter rewarding Royal Irises?. *BioRxiv* doi: 10.1101/184382

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid Dactylorhiza sambucina (L.) *Soò. Proc. Natl. Acad. Sci.* 98, 6253–6255. doi: 10.1073/pnas.111162598

Giurfa, M., Núñez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comparat. Physiol. A* 177, 247–259. doi: 10.1007/BF00192415

Goldberg, E. E., Kohn, J. R., Lande, R., Robertson, K. A., Smith, S. A., and Igic, B. (2010). Species selection maintains self-incompatibility. *Science* 330, 493–495. doi: 10.1126/science.1194513

Goldblatt, P., and Manning, J. C. (2008). *The Iris family: Natural History and Classification*. Portland: Timber Press.

Goldblatt, P., Savolainen, V., Porteous, O., Sostaric, I., Powell, M., Reeves, G., et al. (2002). Radiation in the Cape flora and the phylogeny of peacock irises Moraea (Iridaceae) based on four plastid DNA regions. *Mole. Phylogenet. Evol.* 25, 341–360. doi: 10.1016/S1055-7903(02)00235-X

Gray, S. M., and McKinnon, J. S. (2007). Linking color polymorphism maintenance and speciation. *Trends Ecol. Evol.* 22, 71–79. doi: 10.1016/j.tree.2006.10.005

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Harborne, J. B., and Williams, C. A. (2000). The phytochemical richness of the Iridaceae and its systematic significance. *Annal Di Bot.* 58, 43–50. doi: 10.4462/annbotrm-9061

Imbert, E., Wang, H., Anderson, B., Hervouet, B., Talavera, M., and Schatz, B. (2014a). Reproductive biology and colour polymorphism in the food-deceptive Iris lutescens (Iridaceae). *Acta Bot. Gall.* 161, 117–127. doi: 10.1080/12538078.2014.895419

Imbert, E., Wang, H., Conchou, L., Vincent, H., Talavera, M., and Schatz, B. (2014b). Positive effect of the yellow morph on female reproductive success in the flower colour polymorphic Iris lutescens (Iridaceae), a deceptive species. *J. Evolut. Biol.* 27, 1965–1974. doi: 10.1111/jeb.12451

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-9658(2003)084[1733:trohit]2.0.co;2

Ishii, H. S. (2005). Analysis of bumblebee visitation sequences within single bouts: Implication of the overstrike effect on short-term memory. *Behav. Ecol. Sociobiol.* 57, 599–610. doi: 10.1007/s00265-004-0889-z

Jiang, Y. L., Huang, Z., Liao, J. Q., Song, H. X., Luo, X. M., Gao, S. P., et al. (2018). Phylogenetic analysis of Iris L. *from China on chloroplast TRNL-F sequences. Biol.* 73, 459–466. doi: 10.2478/s11756-018-006360

Katoh, K., Rozewicki, J., and Yamada, K. (2017). MAFFT online service: multiple sequence 149 alignment, interactive sequence choice and visualization. *Brief. Bioinform.* 20(4), 1160–1166. doi: 10.1093/bib/bbx108

Khassanov, F. O., and Rakhimova, N. (2012). Taxonomic revision of the genus Iris L. *(Iridaceae Juss.) for the flora of Central Asia. Stapfia* 97, 174–179.

Kranas, H., Spalik, K., and Banasiak, Ł (2018). *MatPhylobi, 0.1*. Warsaw: University of Warsaw.

Kuraku, S., Zmasek, C., Nishimura, O., and Katoh, K. (2013). Leaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. *Nucl. Acids Res.* 41, 22–28.

Landis, J. B., Bell, C. D., Hernandez, M., Zenil-ferguson, R., McCarthy, E. W., Soltis, D. E., et al. (2018). Evolution of Floral Traits and Impact of Reproductive Mode on Diversification in the Phlox Family (Polemoniaceae). *Mole. Phyl. Evol.* 127, 878–890 doi: 10.1016/j.ympev.2018.06.035

Lavi, R., and Sapir, Y. (2015). Are pollinators the agents of selection for the extreme large size and dark color in Oncocyclus irises? *New Phytol.* 205, 369–377. doi: 10.1111/nph.12982

Little, R. J. (1983). "A review of floral food deception mimicries with comments on floral mutualism," in *Handbook of experimental pollination biology*, eds C. E. Jones, and R. J. Little, (New York: Van Nostrand Reinhold), 294–309.

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comparat. Physiol. A* 177, 1–19. doi: 10.1007/BF00243394

Lyon, D. L. (1973). Territorial and feeding activity of broad-tailed hummingbirds (*Selasphorus platycercus*) in *Iris missouriensis*. *Condor* 75, 346-349. doi: 10.2307/1366178

Maddison, W. P., and Maddison, D. R. (2018). Mesquite: a modular system for evolutionary analysis. *Evolution* 11:5.

Martin, N. H., Sapir, Y., and Arnold, M. L. (2008). The genetic architecture of reproductive isolation in Louisiana Irises: pollination syndromes and pollinator preferences. *Evolution* 62, 740–752. doi: 10.1111/j.1558-5646.2008.00342.x

Martins, T. R., Jiang, P., and Rausher, M. D. (2016). How petals change their spots: cis-regulatory re-wiring in Clarkia (Onagraceae). *New Phytol.* 216(2), 510–518. doi: 10.1111/nph.14163

Mathew, B. (1989). *The iris. Portland, Ore*. United Kingdom: Timber Press.

Monty, A., Saad, L., and Mahy, G. G. (2006). Bimodal pollination system in rare endemic Oncocyclus irises (Iridaceae) of Lebanon. *Canad. J. Bot.* 84, 1327–1338. doi: 10.1139/b06-081

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Ng, J., and Smith, S. D. (2018). Why are red flowers so rare? Testing the macroevolutionary causes of tippiness. *J. Evolut. Biol.* 31, 1863–1875. doi: 10.1111/jeb.13381

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., et al. (2013). Caper: comparative analyses of phylogenetics and evolution in R.R. *package version 0.5.2*.

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature* 40, 877–884. doi: 10.1038/44766

Parachnowitsch, A. L., Manson, J. S., and Sletvold, N. (2018). Evolutionary ecology of nectar. *Anna. Bot.* 123, 247–261. doi: 10.1093/aob/mcy132

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* 20, 289–290. doi: 10.1093/bioinformatics/btg412

Pellegrino, G. (2015). Pollinator limitation on reproductive success in Iris tuberosa. *AOB Plants* 7:lu089. doi: 10.1093/aobpla/plu089

Pellegrino, G., Bellusci, F., and Palermo, A. M. (2017). Functional differentiation in pollination processes among floral traits in Serapias species (Orchidaceae). *Ecol. Evolut.* 7, 7171–7177. doi: 10.1002/ece3.3264

QGIS Development Team (2020). *QGIS Geographic Information System. Open Source Geospatial Foundation Project*. Available online at: http://qgis.osgeo.org

Rausher, M. D. (2008). Evolutionary Transitions in Floral Color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Roddy, A. B. (2019). A physiological approach to the ecology and evolution of flowers. *Int. J. Plant Sci.* 9, 944–953. doi: 10.1101/539668

Rodríguez-Gironés, M. A., and Santamaría, L. (2004). Why are so many bird flowers red? *PLoS Biol.* 2:e350. doi: 10.1371/journal.pbio.0020350

Roguz, K., Bajguz, A., Golebiewska, A., Chmur, M., Hill, L., Kalinowski, P., et al. (2018). Functional Diversity of Nectary Structure and Nectar Composition in the Genus Fritillaria (Liliaceae). *Front. Plant Sci.* 9:1246. doi: 10.3389/fpls.2018.01246

Roguz, K., Bajguz, A., Chmur, M., Gołębiewska, A., Roguz, A., and Zych, M. (2019). Diversity of nectar amino acids in the Fritillaria (Liliaceae) genus: ecological and evolutionary implications. *Sci. Rep.* 9:15209. doi: 10.1038/s41598-019-5117051174

Roy, R., Schmitt, A. J., Thomas, J. B., and Carter, C. J. (2017). Review: Nectar biology: From molecules to ecosystems. *Plant Sci.* 262, 148–164. doi: 10.1016/j.plantsci.2017.04.012

Royal Botanic, and Gardens, K. (2020). *World checklist of selected plant families*. Peradeniya: Royal Botanic.

Sapir, Y., and Shmida, A. (2002). Species concepts and ecogeographical divergence of Oncocyclus irises. *Isr. J. Plant Sci.* 50, S119–S127.

Sapir, Y., Shmida, A., Fragman, O., and Comes, H. P. (2002). Morphological variation of the Oncocyclus irises (Iris: Iridaceae) in the southern Levant. *Botan. J. Linn. Soc.* 139, 369–382. doi: 10.1046/j.1095-8339.2002.00067.x

Sapir, Y., Shmida, A., and Ne'eman, G. (2005). Pollination of Oncocyclus irises (Iris: Iridaceae) by night-sheltering male bees. *Plant Biol.* 7, 417–424. doi: 10.1055/s-2005837709

Sapir, Y., Shmida, A., and Ne'eman, G. (2006). Morning floral heat as a reward to the pollinators of the Oncocyclus irises. *Oecologia* 147, 53–59. doi: 10.1007/s00442-005-0246-6

Schaefer, H., and Ruxton, G. (2011). "Floral communication and pollination," in *Plant-animal communication*, eds H. Schaefer, and G. D. Ruxton, (Oxford: Oxford University Press).

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Simpson, B. B., and Neff, J. L. (1983). "Evolution and diversity of floral rewards," in *Handbook of Experimental Pollination Biology*, eds C. E. Jones and R. J. Little (New York, NY: Van Nostrand Reinhold), 142–159.

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Agren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Smith, S. D. (2015). Tempo and mode of flower color evolution. *Am. J. Bot.* 102(7), 1014–25. doi: 10.3732/ajb.1500163

Smithson, A., and MacNair, M. R. (1997). Negative frequency- dependent selection by pollinators on artificial flowers with- out rewards. *Evolution* 51, 715–723. doi: 10.2307/2411148

Souto-Vilarós, D., Vuleta, A., Jovanović, S. M., Budečević, S., Wang, H., Sapir, Y., et al. (2018). Are pollinators the agents of selection on flower colour and size in irises? *Oikos* 127, 834–846. doi: 10.1111/oik.04501

Spaethe, J., Streinzer, M., and Paulus, H. F. (2010). Why sexually deceptive orchids have colored flowers. *Commun. Integr. Biol.* 3, 139–141. doi: 10.4161/cib.3.2.10333

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. Unite. Stat. Am.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Streisfeld, M. A., and Rausher, M. D. (2011). Poulation genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* 65, 629–642. doi: 10.1111/j.1558-5646.2010.01165.x

Sutherland, S. D., and Vickery, R. K. Jr. (1993). On the relative importance of floral color, shape, and nectar rewards in attracting pollinators to Mimulus. *Great Basin Natural.* 53, 107–117.

Szczepanek, R. (2012). Quantum GIS – wolny i otwarty system informacji geograficznej. *Czasopismo Techn.* 4, 171–182.

Talavera, G., and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *System. Biol.* 56, 564–577. doi: 10.1080/10635150701472164

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Teixido, A. L., Barrio, M., and Valladares, F. (2016). Size Matters: Understanding the Conflict Faced by Large Flowers in Mediterranean Environments. *Botan. Rev.* 82, 204–228. doi: 10.1007/s12229-016-91689168

Tucić, B., Tomic, V., Avramov, S., and Pemac, D. (1998). Testing the adaptive plasticity of Iris pumila leaf traits to natural light conditions using phenotypic selection analysis. *Acta Oecol.* 19, 473–481. doi: 10.1016/s1146-609x(99)80001-0

Vereecken, N. J., Dorchin, A., Dafni, A., Hötling, S., Schulz, S., and Watts, S. (2013). A pollinators' eye view of a shelter mimicry system. *Anna. Bot.* 111, 1155–1165. doi: 10.1093/aob/mct081

Vereecken, N. J., and Schiestl, F. P. (2009). On the roles of colour and scent in a specialized floral mimicry system. *Anna. Bot.* 104, 1077–1084. doi: 10.1093/aob/mcp208

Wang, H., Talavera, M., Min, Y., Flaven, E., and Imbert, E. (2016). Neutral processes contribute to patterns of spatial variation for flower colour in the Mediterranean Iris lutescens (Iridaceae). *Anna. Bot.* 117, 995–1007. doi: 10.1093/aob/mcw036

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Waser, N. M., and Price, M. V. (1981). Pollinator choice and stabilizing selection for flower color in Delphinium nelsonii. *Evolution* 35, 376–390. doi: 10.2307/2407846

Watts, S., Sapir, Y., Segal, B., and Dafni, A. (2013). The endangered Iris atropurpurea (Iridaceae) in Israel: honey-bees, night-sheltering male bees and female solitary bees as pollinators. *Anna. Bot.* 111, 395–407. doi: 10.1093/aob/mcs292

Wesselingh, R. A., and Arnold, M. L. (2000). Nectar production in Louisiana iris hybrids. *Int. J. Plant Sci.* 161, 245–251. doi: 10.1086/314252

Wessinger, C. A., and Rausher, M. D. (2012). Lessons from flower colour evolution on targets of selection. *J. Experim. Bot.* 63, 5741–9. doi: 10.1093/jxb/ers267

Wessinger, C. A., and Rausher, M. D. (2014). Predictability and irreversibility of genetic changes associated with flower color evolution in Penstemon barbatus. *Evolution* 68, 1058–1070. doi: 10.1111/evo.12340

Wester, P., and Lunau, K. (eds) (2017). "Plant-Pollinator Communication," in *How Plants Communicate with their Biotic Environment*, (Netherland: Elseviere), 225–257. doi: 10.1016/bs.abr.2016.10.004

Willmer, P. (2017). *Pollination and floral ecology*. New Jersey: Princeton University Press.

Wilson, C. (2006). Patterns in Evolution in Characters That Define Iris Subgenera and Sections. *Aliso* 22, 425–433. doi: 10.5642/aliso.20062201.34

Wilson, C. A. (2004). Phylogeny of Iris based on chloroplast matK gene and trnK intron sequence data. *Mole. Phylogenet. Evolut.* 33, 402–412. doi: 10.1016/j.ympev.2004.06.013

Wilson, C. A. (2009). Phylogenetic relationships among the recognized series in Iris section Limniris. *System. Bot.* 34, 277–284. doi: 10.1600/036364409788606316

Wilson, C. A., Padiernos, J., and Sapir, Y. (2016). The royal irises (Iris subg. *Iris sect. Oncocyclus): Plastid and low-copy nuclear data contribute to an understanding of their phylogenetic relationships*. *Taxon* 65, 35–46. doi: 10.12705/651.3

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Intrafloral Color Modularity in a Bee-Pollinated Orchid

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Flower color has been studied in different ecological levels of organization, from individuals to communities. However, it is unclear how color is structured at the intrafloral level. In bee-pollinated flowers, the unidirectional gradient in color purity and pollen mimicry are two common processes to explain intrafloral color patterns. Considering that floral traits are often integrated, usually reflecting evolutionary modules under pollinator-mediated selection, we hypothesize that such intrafloral color patterns are structured by intrafloral color modules as perceived by bee color vision system. Here, we studied the tropical bee-pollinated orchid *Cattleya walkeriana*, given its intrafloral color complexity and variation among individuals. Considering bee color vision, we investigated if intrafloral color modules arose among intrafloral patches (tip or base of the sepals, petals, and labellum). We expected a separate color module between the labellum patches (the main attractive structure in orchids) and petals and sepals. We measured the color reflectance and calculated the photoreceptor excitation, spectral purity, hue, and the chromatic contrast of the floral structures in the hexagon color model. Spectral purity (saturation) was higher in the labellum tip in comparison to petals and sepals, generating a unidirectional gradient. Labellum base presented a less saturated yellow UV-absorbing color, which may reflect a pollen mimicry strategy. *C. walkeriana* presented three intrafloral color modules corresponding to the color of petals and sepals, the color of the labellum tip, and the color of labellum base. These color modules were unrelated to the development of floral structures. Given the importance of intrafloral color patterns in bee attraction and guidance, our results suggest that intrafloral patterns could be the outcome of evolutionary color modularization under pollinator-mediated selection.

Keywords: bees, color signaling, color properties, integration, Orchidaceae

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INTRODUCTION

Floral color is one of the most important signals in plant-pollinator communication, usually increasing pollinators efficiency in detecting flowers in nature and improving their efficacy in obtaining floral resources and pollinate the flower (Wester and Lunau, 2017). Through the visual communication between pollinators and plants, selective pressures can be imposed,

generating a coevolutionary process. In that process, plants will adapt to the perception of pollinators, and pollinators will adapt to floral signals. These processes can generate floral color patterns structured on different ecological organization levels, from communities (Shrestha et al., 2014; Bergamo et al., 2018; Camargo et al., 2019), populations (Mascó et al., 2004; Newman et al., 2012), to the inter-individual level within a population, i.e., color polymorphisms (Bergamo et al., 2016; Jiménez-López et al., 2019; Aguiar et al., 2020). Regardless of the organization level, most studies describe color properties such as petal spectral purity and hue as a single value per flower (Rohde et al., 2013; Shrestha et al., 2013; Koethe et al., 2016). However, a single flower may display distinct colors, creating intrafloral color patterns that attract and guide pollinators (Lunau et al., 1996). Generally, intrafloral color variation in angiosperms is a result from differential pigment accumulation regulated by tissue-specific transcription during the process of pigment biosynthesis (Martins et al., 2017). Despite understudied, intrafloral color patterns are especially common in bee-pollinated flowers (Camargo et al., 2019), and are important features favoring legitimate visits (Medel et al., 2003; Leonard and Papaj, 2011; Papiorek et al., 2016).

There are two non-exclusive general hypotheses to explain the evolution of intrafloral color patterns in bee-pollinated flowers, both related to the selective pressures imposed by pollinator perception and behavior. The first is the saturation gradient, which corresponds to a centripetal increase in spectral purity from flower periphery to the center. Bees orientate themselves toward the center of the flower following a gradient of centripetally increasing color saturation produced by floral guides (Lunau, 1990). When the spectral purity (saturation) of the corolla is lower than the spectral purity of the guides, bumblebees and honeybees innately react to guides, approaching and inspecting them (Lunau, 1990; Lunau et al., 2009). Several structures are examples of intrafloral guides such as spots (Thomas et al., 2009), bull's eye patterns (Papiorek et al., 2016) and pigmentation over the petal vasculature or veins, often referred to as resource guides (Whitney et al., 2013). The second hypothesis to explain intrafloral color patterns is related to the "pollen mimicry" strategy, based on the fact that many flowering plants display pollen- and stamen-like structures, enhancing or replacing the visual signals associated with pollen itself (Heuschen et al., 2005; Lunau et al., 2017). The strategy consists in presenting yellow structures or areas of similar color to those of anthers and pollen. Bees react to yellow UV-absorbing areas in natural or artificial flowers and prefer those over flowers presenting no such signals (Papiorek et al., 2016; Telles et al., 2020). The presence of these yellow signals can potentially increase plant fitness, by inducing visits (Lunau, 2006; Duffy and Johnson, 2015; Lunau et al., 2017).

Intrafloral color patterns are probably the outcome of a complex interplay among different selective pressures on flower pigments, including those exerted by the preferences of pollinators (Schemske and Bradshaw, 1999; Medel et al., 2003; Caruso, 2004; Papiorek et al., 2016; Caruso et al., 2019), abiotic factors, and genetic and developmental constraints (Ashman and Majetic, 2006; Klingenberg, 2014; Bergamo et al., 2018). As pollinators can act as an important source of natural selection, Raissa Berg proposed that correlation among reproductive traits would generate floral integration, as a consequence of the selective pressures on traits that promoted successfully pollination (Berg, 1960; Alcantara et al., 2013; Armbruster et al., 2014; Fornoni et al., 2016). However, selection could act differently on distinct floral structures, due to different functions, generating distinct trait modules within flowers ("intrafloral modularity") (Armbruster et al., 1999, 2014; Pérez et al., 2007; Ordano et al., 2008; Baranzelli et al., 2014; Dellinger et al., 2019). Intrafloral modularity is the tendency of some floral traits to covary among themselves while being independent of other traits (Armbruster and Wege, 2019). Moreover, pollination efficacy is thought to increase as a result of intrafloral modularity (Armbruster and Wege, 2019). The same mechanisms that generates intrafloral modularity could be applied to the comprehension of intrafloral color patterns.

Considering the interaction with pollinators, different color areas within a flower could attract pollinators first from a distance, and then guide their approach at a close range (Lunau, 1993). In doing so, complex intrafloral signals could be decoupled in different modules that maximize plant fitness through consistent pollinator orientation. Taking into consideration the two intrafloral color variation hypotheses explained above, pollinator-mediated integration (and further modularization in the different floral structures) could be the mechanism behind the centripetal increase in color saturation. Integration in color hue could also lead to yellow UV-absorbing modules, leading to a pollen mimicry pattern.

In orchids, floral color often shows intraspecific polymorphism and many orchids present nectar guides and differences in color between floral parts (Darwin, 1877; Aguiar et al., 2012; Sletvold et al., 2016; Pansarin et al., 2018). Furthermore, orchid flowers present a specialized structure, the labellum, a modified petal which is usually distinct from petals and sepals regarding both color and morphology (Darwin, 1877; van der Pijl and Dodson, 1966; Fay and Chase, 2009). In this case, while petals and sepals attract the pollinators from long distances, the labellum is related to pollinator attraction and behavior in short distances (van der Pijl and Dodson, 1966; Fay and Chase, 2009). Given their potential different functions, sepals have evolved semi-independently from the petals, and the labellum has evolved semi-independently from petals (Mondragón-Palomino and Theißen, 2009). In addition, there was a phylogenetic conservatism during labellum evolution in comparison to the high lability of other petals and sepals in a specific orchid group (*Cirrhopetalum* alliance), revealing that pollinator-mediated selection could have a role in the modular evolution of orchid flowers (Hu et al., 2019), which could also influence intrafloral color patterns. Also, the hybrid orchid *Cattleya* "KOVA," presents a spatiotemporal variation in pigments accumulation during floral development, regulated by three different transcription factors (Li et al., 2020). As a result, a low accumulation of pigments in sepals and lateral petals promote a pale pink color, while a high accumulation of cyanine pigments in the labellum tip and of carotenoids in the labellum base promote purple-red and yellow colorations,

respectively (Li et al., 2020). Therefore, considering different ecological functions and the differences in pigment accumulation in floral tissues, it would be expected that the perceived intrafloral pattern in *Cattleya* sp. is a result of intra-floral color modularity. Although intraspecific color variation is well documented in orchids (Gigord et al., 2002; Juillet and Scopece, 2010; Ackerman et al., 2011; Kagawa and Takimoto, 2016; Aguiar and Pansarin, 2019; Aguiar et al., 2020), intrafloral color variation has received little attention.

In this study, we investigated the intrafloral color patterns of the tropical bee-pollinated orchid *Cattleya walkeriana* Gardner focusing on interindividual variation of hue and spectral purity taking into account the bee-color vision system. Considering the differential morphology of floral structures and their known functions, we expected to find an increasing pattern of saturation toward the flower center, with sepals and petals less saturated than the labellum. We also describe the presence of a yellow UV-absorbing center, which could work as floral guide to bees during approach, in a pollen mimicry mechanism. We also expected to find less color variation among plants in the center of their flowers when compared to their peripheries. Finally, we expected to find three intrafloral color modules corresponding to the color of petals and sepals, the color of the labellum tip and the color of labellum base.

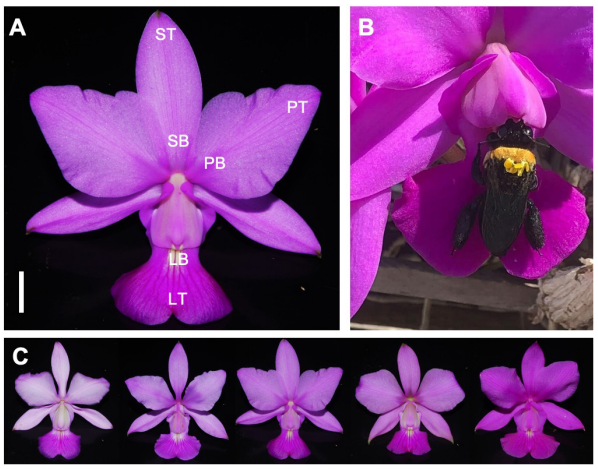


FIGURE 1 | Flowers of *Cattleya walkeriana*. **(A)** intrafloral color patches sampled; **(B)** a visit of a bee pollinator carrying some pollinia; **(C)** color variation among sampled individuals. PB, petal base; PT, petal tip; SB, sepal base; ST, sepal tip; LB, labellum base; LT, labellum tip. Bar = 1 cm. Photos **(A,C)**: Vinícius L. G. Brito; photo **(B)**: courtesy of Joaquim Barreto Carneiro Filho.

MATERIALS AND METHODS

Plant Species and Growth Conditions

Cattleya walkeriana Gardner (Orchidaceae: Laeliinae) is a widespread bee-pollinated orchid (Silva and Milaneze-Gutierre, 2005) found on rocks or trees in areas near lakes, rivers, and swamps in different regions of Cerrado, the Brazilian savanna (Faria et al., 2002). Flowers present a color pattern with pinkish to dark pink sepals and petals, and a dark pink labellum with a yellow area on its base (**Figure 1**). Some species of the same genus have been repeatedly reported to be self-compatible, pollinator-dependent and nectarless, deceiving bees and hummingbirds during the pollination process (Smidt et al., 2006; Caballero-Villalobos et al., 2017). Despite some records of *C. walkeriana* flowers visited by bees (**Figure 1B**), detailed information about the reproduction of this species is still unknown. This orchid bloom mostly from May to July and the duration of flower anthesis is relatively long (from 7 to 10 days). Breeding systems tests revealed that *C. walkeriana* is self-compatible but depends on pollinators to set fruits (**Supplementary Table S1**). Spontaneous selfing and apomixis treatments did not produce any fruit, whereas hand-selfing and hand-crossing treatments resulted in 34% and 54% fruit-set, respectively (**Supplementary Table S1**).

All the plants used in this study were collected during the flowering period of 2011 in four natural areas of Goiás State, Brazil. Due to common predatory orchid collection practice, sampling areas are available only under request. Plants were collected at least 10 m apart to each other and were kept in the same growth conditions in the greenhouse of Orquidário Paranaíba, in Itumbiara municipality, Goiás State, Brazil. Plants were cultivated in clay pots (20 cm in diameter and 15 cm in depth) using tree barks as substrate. To reduce the possible effects of environment on color differences (Schemske and Bierzychudek, 2001; Dafni et al., 2020), individuals were cultivated in the same natural light and temperature conditions and were watered at least twice a day.

Reflectance Measurements

To study floral color properties as perceived by bees, we measured the spectral reflectance of the tip and base of different floral structures, which we named as floral patches throughout this text, from 30 plant individuals (one flower per individual) during the flowering season of 2015. Floral structures were divided in sepals, petals, and labellum (**Figure 1A**). All the measurements were performed in recently open flowers, following the sequence from sepal to labellum in each plant, measuring it at the tip and the base of each floral structure (**Figure 1**), totaling 180 measurements. Spectral reflectance curves were collected using a portable spectrophotometer with a built-in light source (Jaz; Ocean Optics Inc., Dunedin, FL, United States; light source range from 189.91 nm until 896.99 nm) coupled with an optic fiber reflection probe (R400-7-UV-VIS Jaz; Ocean Optics Inc., Dunedin, FL, United States). To calibrate the spectrophotometer, we used a white diffuse reflectance pattern (WS-1, Ocean Optics Inc., Dunedin, FL, United States) and covered the spectrophotometer entrance as black standard. All the measurements were made at an angle of 90° with the probe positioned 2 mm from the sample.

Color Modeling, Color Properties and Color Distances

We estimated the color locus occupied by each floral patch (tip and base of each floral structure) in the hexagon color space (Chittka, 1992). To that, we used the floral patch reflectance, a

green leaf standard as the background (Chittka and Kevan, 2005), and a daylight illumination (D65; Wyszecki and Stiles, 1982), together with the spectral sensitivity of *Bombus terrestris* (Peitsch et al., 1992). From that, we estimated the excitation values of each the UV, blue and green photoreceptors and posteriorly the position (X and Y coordinate values) on the hexagon color space (Chittka, 1992). Once we build the model and estimated the color locus of each floral patch, we measured the color properties, which are hue (i.e., the pure state of color, derived from the different pigment composition in the flower, which leads to what we know as different colors such as red or blue) and the relative spectral purity (i.e., the hue intensity, related to different pigment concentration in the flower, originating dark and light versions of the same hue; Dormont et al., 2019). We also calculated the color contrast against the background (i.e., the color difference between an object and its background).

In the hexagon model, color hue can be measured as the angle formed between the color locus and any axis chosen arbitrarily (Chittka, 1992; Shrestha et al., 2014). Therefore, we measured color hue as the angle between the *x*-axis of the hexagon model and the line crossing the hexagon center and the color loci (Chittka, 1992). Spectral purity was obtained considering the perceptual distance between the locus of each floral patch measured and the background, divided by the distance between the corresponding spectral locus and the maximal spectral purity at the same locus (Lunau et al., 1996). Finally, color contrast against the background was measured as the distance between the color locus and the central point of the color hexagon, which represents the locus of the standard green background (Chittka, 1992).

To estimate color variation within patches among plants, we calculated the distance, in hexagon units, among the color loci belonging to the same floral patch. In the hexagon model, color distances may be estimated in two different ways that also have different biological meanings. Euclidian color distances between loci is a proxy for chromatic contrast, while the angular distance between loci corresponds to the difference in color hue. We are aware that bees behave differently to colors on different hexagons subsets, in a way that similar Euclidean distances might not elicit similar discrimination responses by bees (Dyer and Chittka, 2004; Telles and Rodríguez-Gironés, 2015), yet the bee hexagon color space model is a simple and reliable tool to access the kind of information we are aiming to evaluate in this study (Gawryszewski, 2017).

Data Analyses

As spectral purity and contrast against background were highly correlated in our sample ($r_{Pearson}$ = 0.94; df = 178; $p < 0.001$), we decided to perform the statistical analyses only using the spectral purity because it is directly related to the saturation gradient hypothesis. To analyze the intrafloral color patterns of *C. walkeriana*, we compared the spectral purity of each floral patch using a linear mixed-effect model. In this model, the spectral purity was considered as the response variable, while floral structures (sepal, petal, and labellum), the position of reflection curve measurement (tip and base) as well as the interaction among these factors were considered the explanatory variables. The identity of plant individuals was considered a random effect. Afterward, we ran *post hoc* pairwise multiple comparison *t* tests between all floral patches with false discovery rate (FDR) at a significance level of 0.05 (Benjamini and Hochberg, 1995). We also compared the values of color hue among floral patches using a circular one-way ANOVA. Unfortunately, the current tools to perform circular ANOVA do not allow interaction terms, and thus, we could not include an interaction between floral structures and the position of reflection curve measurement. Therefore, color hue was considered the response variable, and the color patches (sepal tip, sepal base, petal tip, petal base, labellum tip, and labellum base as levels) as the explanatory variables. We used the Watson's two-sample test of homogeneity for *post hoc* comparisons. As such test does not allow any correction for multiple comparisons, we used a conservative significance level of 0.001.

We used both color distances, Euclidean and angular, to analyze color variation within patches between pairs of plants (totaling 435 comparison pairs). In the first case, we used a linear mixed model considering the squared root of Euclidian distances between loci of the same floral patch as the response variable and, floral structures (sepal, petal, and labellum), the measurement position (tip and base) and the interaction between them as explanatory variables. The plant pair were considered a random term. Afterward, we ran a pairwise multiple comparisons *t*-test with FDR procedures at significance level of 0.05 (Benjamini and Hochberg, 1995). We back transformed the values to the Euclidian distances when building the plot corresponding to this analysis for better visualization. The angular distances between the loci of pairs of patches were analyzed using a circular one-way ANOVA. The angular color distance between loci of the same floral patch was considered as the response variable, and the color patches as the explanatory variable. We used the Watson's two-sample test of homogeneity for *post hoc* comparisons with a conservative significance level of 0.001. We validated all the previous models by visually checking whether the residuals were evenly distributed around the fitted values.

Finally, we used the covariance-ratio (CR) test (Adams, 2016) to explicitly test the hypothesis that intrafloral color patterns of *C. walkeriana* correspond to three intrafloral color modules, i.e., the color of petals and sepals, the color of the labellum tip and the color of labellum base. The CR uses pairwise covariances to quantify modular structures and ranges from zero to positive values. A low CR value (i.e., between zero and one) indicates relatively less covariation among modules than that found within modules, characterizing a more modular structure in the data. On the other hand, a higher CR value describe higher covariation among modules. In this analysis, we used the normalized UV, blue and green photoreceptor excitation values as variables rather than X and Y coordinates in the hexagon because photoreceptor excitation values have direct biological meaning related to color as perceived by bees. The significance of the CR value found was evaluated by 10000 permutations in which photoreceptor excitation values for each flower patch were randomly assigned to one of the three designated color modules. The proportion of permuted CR values lower than the original

was treated as an estimate of the significance level of the test (Adams, 2016).

All analyses were performed in R (ver. 3.6.2; R Development Core Team, 2020), using the packages *pavo* (Maia et al., 2019), *circular* (Agostinelli and Lund, 2013), *lme4* (Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2017), *geomorph* (Adams and Otárola-Castillo, 2013), and *ggplot2* (Wickham, 2016).

RESULTS

Floral Color Properties

Reflectance of the tip and base of sepals and petals, as well as those of the tip of the labellum were similar, reflecting light in blue and red wavelengths, while labellum base reflected mostly in green and red wavelengths (**Supplementary Figure S1**). When considering the hexagon space, floral patches occupied different color sections (**Supplementary Figure S2**). The loci of sepals and petals were in the blue section of the hexagon, while labellum tip loci fell in the UV-blue section and labellum base loci were in blue-green section (**Supplementary Figure S2**).

There was a significant interaction between floral structure (sepal, petal, or labellum) and position of reflection curve measurement (tip or base) (F = 5.728; df = 2; $p < 0.01$; **Supplementary Table S2** and **Figure 2**). Sepals and petals presented the lowest purity irrespective of position (**Supplementary Table S2**). In general, the labellum presented spectral purity 56% higher than petals and sepals regardless the measurement position (**Supplementary Table S2** and **Figure 2**). Thus, considering the different floral structure identity, spectral purity increased from flower periphery to center, with labellum presenting higher spectral purity than sepals and petals. However, contrary to our expectations, taking in consideration the variation within floral structure, petal base exhibited lower purity than petal tip, and labellum tip presented higher purity than labellum base (**Supplementary Table S2** and **Figure 2**). Considering hue on the hexagon space, sepals and petals did not differ in this color property, while the labellum presented a distinct hue (**Figure 3** and **Supplementary Table S2**). Moreover, hue differed between labellum tip and base (**Figure 3** and **Supplementary Table S2**).

Color Distances Among Plant Individuals

The interaction between floral structure and position of reflection curve measurement explained color Euclidian distances among pairs of plant individuals (F = 30.681; df = 2; $p < 0.001$). In general, sepals, petals and labellum tips presented Euclidian distance among individuals from 0.07 to 0.09 hexagon units (**Supplementary Table S3**). However, Euclidian distance in labellum bases among individuals could reach 0.12 hexagon units. Therefore, Euclidian distance among pairs (i.e., color contrast) of plant individuals were 58% higher in labellum bases than in any other combination of floral structure and position (**Figure 4A** and **Supplementary Table S3**).

The angular color distance among plant individuals differed between floral structure and position of reflection curve measurement (F = 65.7; df = 5; $p < 0.001$). In general, angular color distance among individuals in sepals and petals varied from 14° to 19°, while angular color distance among individuals were approximately 9° for both labellum tip and base. The mean angular distance (i.e., color hue difference) of the labellum among plant individuals were lower than the mean angular distance of any other combination of floral structure and position (**Figure 4B** and **Supplementary Table S3**).

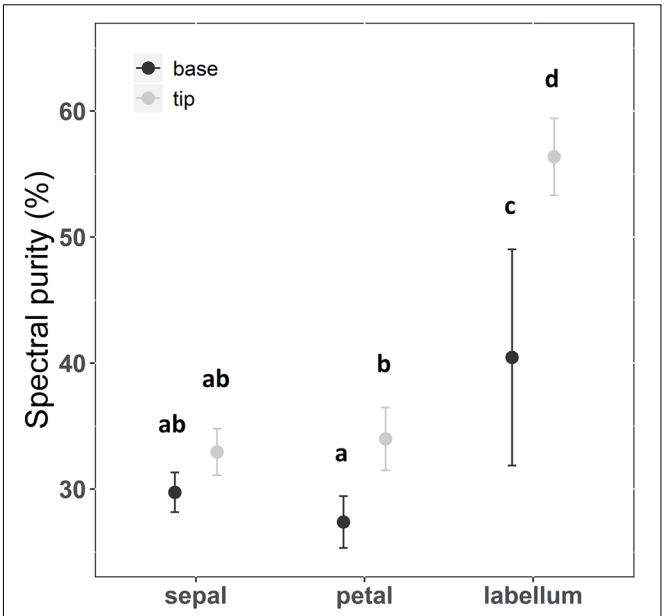


FIGURE 2 | Spectral purity of floral patches of *Cattleya walkeriana*. Dots indicate the mean value and bars the 95% confidence interval. Different letters show statistical difference after pairwise multiple comparison among floral patches, with a false discovery rate at a significance level of 0.05. N = 30 individuals.

Intrafloral Color Modularity

The CR value found for the three proposed color modules in *C. walkeriana* was 0.42 (confidence interval: 0.37–0.76; $p < 0.001$) indicating that intrafloral color patterns correspond to three independent color modules.

DISCUSSION

Many flowers present intrafloral color pattern, and such pattern has a role in attraction and guidance of pollinators to the flowers' resource (Medel et al., 2003; Leonard and Papaj, 2011; Papiorek et al., 2016). Here we investigated the intrafloral color pattern as well as their variation among plants of *C. walkeriana*, a bee-pollinated orchid (Silva and Milaneze-Gutierre, 2005), which depends on pollinators to set their fruits (**Supplementary Table S1**). Therefore, intrafloral color patterns may play a role in *C. walkeriana* reproduction if such pattern affects bee attraction. As we expected, the flowers of this orchid presented a centripetally increasing spectral purity, being petals and sepals (periphery) less saturated than the labellum (center). Also, the yellow UV-absorbing hue of labellum base showed the lowest

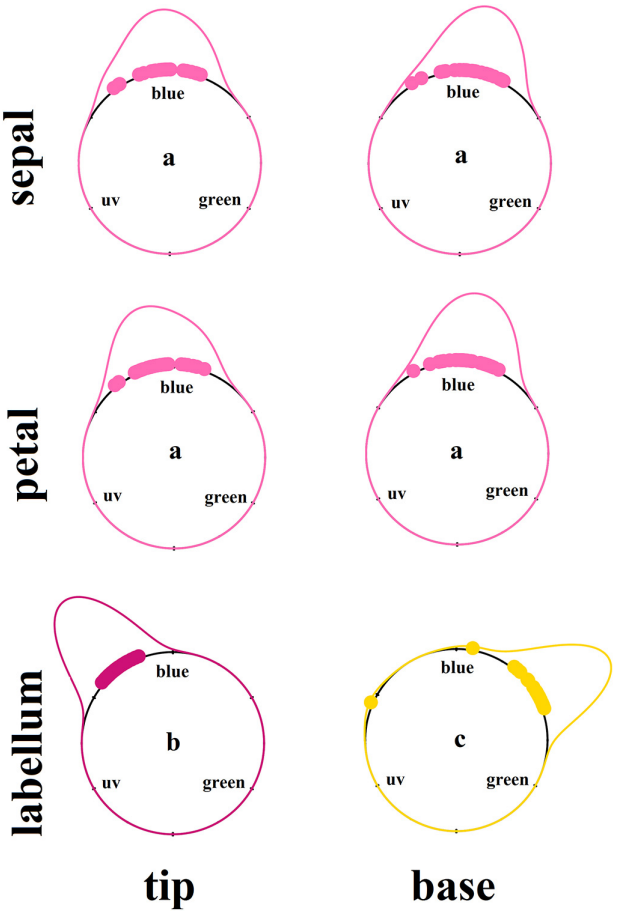


FIGURE 3 | Kernel density estimation of the color hue of floral patches of *Cattleya walkeriana*. Dots indicate the raw hue values of each color loci. Density curves color represent the floral structure as perceived by the human eyes. Letters indicate statistical difference after Watson's two-sample test of homogeneity, at significance level of 0.001. $N = 30$ individuals.

variation among floral patches. Several bee-pollinated flowers are yellow UV-absorbing, a color often used by bees to locate resources (Heuschen et al., 2005; Lunau et al., 2017). Finally, we found modularity in intrafloral color, being the colors of sepals and petals, labellum tip, and labellum base distinct modules as perceived by bees. We expected to find this color modularity as, in orchids, the labellum is often the main floral structure in terms of pollinator attraction and pollinator orientation, usually presenting different features compared to the other floral elements (van der Pijl and Dodson, 1966; Fay and Chase, 2009; Armbruster and Wege, 2019).

The great majority of the bee-pollinated *Cattleya* spp. studied so far are food deceptive (Smidt et al., 2006; Silva-Pereira et al., 2007; Caballero-Villalobos et al., 2017). If this is also the case of *C. walkeriana*, the intrafloral color variation pattern found here could be even more advantageous in this rewardlessness scenario. In this sense, the intrafloral color pattern generated by color modularity would improve generalized food deceptive exploitation of pollinators based on their innate cognitive preferences (Jersáková et al., 2006; Johnson and Schiestl, 2016).

Centripetal Increase on Color Spectral Purity

Although petals and labellum presented higher spectral purity at their tip than at their base, there was a general increase of spectral purity in *C. walkeriana* from the periphery to the center of the flower, peaking at the labellum. This pattern could be a consequence of selective pressure imposed by pollinators, as it is linked to a higher attraction and a more effective guidance of pollinators to floral reproductive structures. Spectral purity has been demonstrated to play a role on bee attraction and guidance when approaching and landing on flowers (Rohde et al., 2013), likely favoring correct placement and pickup of pollen on the bee body. Moreover, orchid floral resources are usually located at the labellum in center of the flower (Fay and Chase, 2009). A centripetal increase of color spectral purity could enhance pollination service of effective pollinators in the studied orchid, even in the case when flowers offer no reward at all. Some pollinating bees have innate preference for colors with higher spectral purity (Lunau et al., 1996) and such preference could generate the selective pressures that modulate the observed intrafloral color pattern in *C. walkeriana*. Contrary to our expectations, the petals tip presented higher spectral purity than the base, with a small decrease from the periphery (34.0%) to the center (24.7%). However, as the spectral purity of the labellum is much higher (40.5–56.4%) than the petals, the small variation in spectral purity within sepals and petals should not influence bees' attraction toward the labellum. Further behavioral tests would be useful to support this hypothesis. Within the labellum, the very center of the flower (labellum base) also showed lower spectral purity in relation to labellum tip (although this saturation was still higher in comparison with petals and sepals). A low saturation in labellum base when compared to tip was unexpected by our former hypothesis that intrafloral color saturation should increase centripetally (Lunau, 1990). However, contrasting with the difference in spectral purity at the petals, the variation found at the labellum may be explained by another color constraint in central floral elements: pollen mimicry in the labellum base.

Pollen Mimicry

The labellum base, although presenting lower spectral purity in comparison with labellum tip, presented a completely different hue from the other floral parts. Moreover, color hue variation among individuals was exceptionally low within this floral patch when compared to sepals and petals. In other words, flowers of *C. walkeriana* showed a strong color hue constraint in their labellum base no matter their spectral purity. This is common in specialized bee-pollinated flowers, which usually present less diverse inner colors when compared to the peripheral colors (Heuschen et al., 2005). In *C. walkeriana*, the labellum base is yellow UV-absorbent, which is known to be a color category related to pollen mimicry in bee-pollinated flowers (Lunau, 2000). Many insects seek for pollen during flower visit, and the

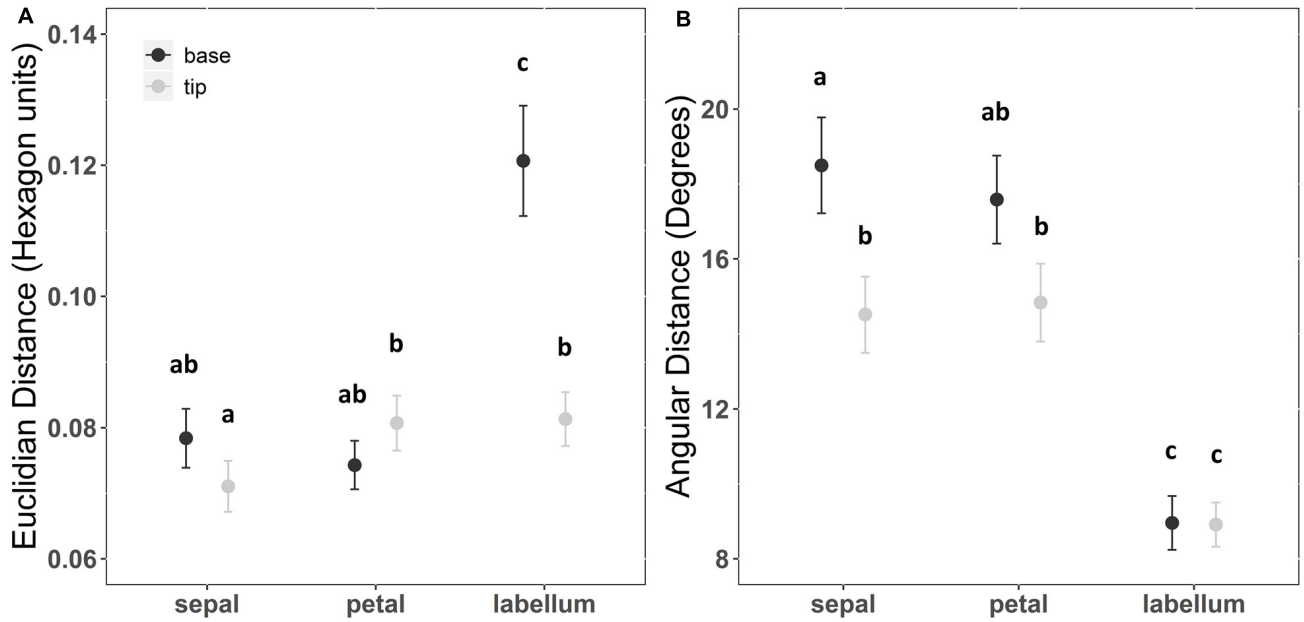


FIGURE 4 | Chromatic contrasts **(A)** and hue differences **(B)** within floral patches among individuals of *Cattleya walkeriana*. Chromatic contrast was measured as the Euclidian distance while hue difference was measured as the angular distance among pairs of color loci in color hexagon. Dots indicate the mean value and bars the 95% confidence interval. Letters above chromatic contrast values indicate statistical differences after a pairwise multiple comparison among floral patches with a false discovery rate at a significance level of 0.05. Letters above angular distance values indicate statistical difference after Watson's two-sample test of homogeneity with a significance level of 0.001.

visual signals of pollen are well conserved across the flowering plants (Lunau, 1995; Lunau et al., 2017). Therefore, presenting yellow UV-absorbent areas or structures may be an attraction advantage to the plants (Johnson and Schiestl, 2016). Thus, although the very center of the flowers of *C. walkeriana* present less color saturation than its immediate surroundings, contrary to the expected centripetal increase of spectral purity, the presence of a yellow UV-absorbent spot, which is very conserved in its hue among individuals, could be a pollen mimicking strategy to increase floral attractiveness for bees regardless its lower saturation.

Intrafloral Color Modularity

Sepals and petals formed a single intrafloral color module, while the labellum tip and labellum base varied independently. The morphology of orchid flowers is regulated by the expression of four different classes of DEF-like genes, even if they are part of the same or different floral whorls. While class 1 and 2 genes are activated in all floral structures, class 3 is activated in the petals and labellum, and class 4 only at the labellum. This promotes the striking intrafloral differentiation found in orchid flowers, and it is assumed to be the genetic base of the vast diversity found in the flowers of Orchidaceae (Mondragón-Palomino and Theißen, 2009). Therefore, the overall similarity in sepals and lateral petals found here could be explained by the common expression of organ identity genes shared between sepals and petals (DEF-like classes 1 and 2) but not in labellum. Also, evidence from the literature shows that the labellum is under differential pollinator-mediated selection in relation to sepals and petals (Mondragón-Palomino and Theißen, 2009; Hu et al., 2019). This was especially strong for the labellum base color. The strong constraint in color hue associated to the high variation in color saturation of the labellum base may have driven the evolution of this floral patch as a completely different intrafloral color module. Contrasting these results with the morphology of sepals, petals, and labellum in orchids, highlights that floral morphology could have evolved semi-independently from the intrafloral color patches, being constrained by different developmental routes of floral pigments and driven by different selective forces.

Patterns of integration and modularity in floral traits among individuals can be determined by different underlying biological processes, such as development, environment, functionality, and genetics (Klingenberg, 2014). In our study, environmental conditions were controlled, and flowers were analyzed in the same developmental stage (i.e., during the floral anthesis). Thus, the intrafloral color modularity found in *C. walkeriana* is not a consequence of environmental variation, yet it represents the evolutionary history experienced by this species. Given the role of color on bee attraction and guidance, color modularity in *C. walkeriana* might be, therefore, the product of selective pressures imposed by pollinators. Armbruster and Wege (2019) proposed that the evolution of both floral and intra-floral modularity is a consequence of the degree of the floral phenotypic specialization more than the ecological specialization. Orchids present flowers phenotypically highly specialized with bilateral symmetry and the pollinator needs to be highly oriented with the fertile

parts to the pollinia be correctly deposited (Darwin, 1877; van der Pijl and Dodson, 1966). This floral phenotypical specialization highlights the role that pollinators had in orchids evolutionary history, including the intrafloral color modules, as shown here. Despite floral integration and modularity have been showed only for morphological floral traits such as the size of structures (Berg, 1960; Armbruster et al., 2014), here we propose that modularity could arise in other floral traits under pollinator-mediated selection, such as color.

CONCLUSION

To our knowledge, our study is the first to show intrafloral color modularity considering the color vision system of the pollinator. This modularization may be the result of selective pressures imposed by the visual preferences of pollinators and the need of consistency in pollinators visits to be efficient. In *C. walkeriana*, both the centripetal increase on color saturation and the yellow UV-absorbent patch of the labellum base create three different intrafloral color modules that are not entirely related to the morphological modules typically found in orchids (Hu et al., 2019). Such modules can be defined as: (1) the less saturated and variable color of sepals and petals, (2) the color of the labellum tip, which showed the highest color saturation, probably as an outcome of the selective pressure imposed by the innate preference of bees for centripetally color saturation in flowers (Lunau, 1990; Rohde et al., 2013), and (3) the color of the labellum base, which shows low hue variation among individuals and is probably an outcome of the selective pressure derived from the innate preference of bees for pollen-like colors (Lunau, 2000; Heuschen et al., 2005). Thus, this work provides an example of how we should take into consideration pollinators' perception and cognition to understand how they could impose selective pressures which shapes intrafloral color patterns evolution. Further studies should investigate floral integration and modularity across distinct traits in order to broaden our understanding of the evolution of intrafloral patterns.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JA and VB wrote the first draft of the manuscript. AM and VB designed the study and collected and analyzed the data. PS, PB, FT, and PO contributed significantly in the discussion of the data and reviewed and contributed to the text of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.589300/full#supplementary-material

REFERENCES

Ackerman, J. D., Cuevas, A. A., and Hof, D. (2011). Are deception-pollinated species more variable than those offering a reward? *Plant Syst. Evol.* 293, 91–99. doi: 10.1007/s00606-011-0430-436

Adams, D. C. (2016). Evaluating modularity in morphometric data: challenges with the RV coefficient and a new test measure. *Methods Ecol. Evol.* 7, 565–572. doi: 10.1111/2041-210X.12511

Adams, D. C., and Otárola-Castillo, E. (2013). geomorph: an R package for the collection and analysis of geometric morphometric shape data. *Methods Ecol. Evol.* 4, 393–399. doi: 10.1111/2041-210X.12035

Agostinelli, C., and Lund, U. (2013). *R Package 'Circular': Circular Statistics (Version 0.4-7)*. Available online at: https://r-forge.r-project.org/projects/circular (accessed September 24, 2020).

Aguiar, J. M. R. B. V., Giurfa, M., and Sazima, M. (2020). A cognitive analysis of deceptive pollination: associative mechanisms underlying pollinators' choices in non-rewarding colour polymorphic scenarios. *Sci. Rep.* 10:9476. doi: 10.1038/s41598-020-66356-66354

Aguiar, J. M. R. B. V., and Pansarin, E. R. (2019). Deceptive pollination of *Ionopsis utricularioides* (Oncidiinae: Orchidaceae). *Flora* 250, 72–78. doi: 10.1016/j.flora.2018.11.018

Aguiar, J. M. R. B. V., Pansarin, L. M., Ackerman, J. D., and Pansarin, E. R. (2012). Biotic versus abiotic pollination in *Oeceoclades maculata* (Lindl.) Lindl (Orchidaceae). *Plant Spec. Biol.* 27, 86–95. doi: 10.1111/j.1442-1984.2011.00330.x

Alcantara, S., de Oliveira, F. B., and Lohmann, L. G. (2013). Phenotypic integration in flowers of neotropical lianas: diversification of form with stasis of underlying patterns. *J. Evol. Biol.* 26, 2283–2296. doi: 10.1111/jeb.12228

Armbruster, W., Di Stilio, V. S., Tuxill, J. D., Flores, T. C., and Velásquez Runk, J. L. (1999). Covariance and decoupling of floral and vegetative traits in nine neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *Am. J. Bot.* 86, 39–55. doi: 10.2307/2656953

Armbruster, W. S., Pélabon, C., Bolstad, G. H., and Hansen, T. F. (2014). Integrated phenotypes: understanding trait covariation in plants and animals. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130245. doi: 10.1098/rstb.2013.0245

Armbruster, W. S., and Wege, J. A. (2019). Detecting canalization and intra-floral modularity in triggerplant (Stylidium) flowers: correlations are only part of the story. *Ann. Bot.* 123, 355–372. doi: 10.1093/aob/mcy184

Ashman, T. L., and Majetic, C. J. (2006). Genetic constraints on floral evolution: a review and evaluation of patterns. *Heredity* 96, 343–352. doi: 10.1038/sj.hdy.6800815

Baranzelli, M. C., Sérsic, A. N., and Cocucci, A. A. (2014). The search for Pleiades in trait constellations: functional integration and phenotypic selection in the complex flowers of *Morrenia brachystephana* (Apocynaceae). *J. Evol. Biol.* 27, 724–736. doi: 10.1111/jeb.12341

Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67:97133. doi: 10.18637/jss.v067.i01

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Berg, R. L. (1960). The ecological significance of correlation pleiades. *Evolution* 14, 171–180. doi: 10.2307/2405824

Bergamo, P. J., Rech, A. R., Brito, V. L. G., and Sazima, M. (2016). Flower colour and visitation rates of *Costus arabicus* support the "bee avoidance" hypothesis for red-reflecting hummingbird-pollinated flowers. *Funct. Ecol.* 30, 710–720. doi: 10.1111/1365-2435.12537

Bergamo, P. J., Telles, F. J., Arnold, S. E. J., and Brito, V. L. G. (2018). Flower colour within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-4205

Caballero-Villalobos, L., Silva-Arias, G. A., Buzatto, C. R., Nervo, M. H., and Singer, R. B. (2017). Generalized food-deceptive pollination in four *Cattleya* (Orchidaceae: Laeliinae) species from Southern Brazil. *Flora* 234, 195–206. doi: 10.1016/j.flora.2017.07.014

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Caruso, C. M. (2004). The quantitative genetics of floral trait variation in *Lobelia*: potential constraints on adaptive evolution. *Evolution* 58, 732–740. doi: 10.1111/j.0014-3820.2004.tb00406.x

Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution* 73, 4–14. doi: 10.1111/evo.13639

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ON: Enviroquest Ltd), 157–196.

Dafni, A., Tzohari, H., Ben-Shlomo, R., Vereecken, N. J., and Ne'eman, G. (2020). Flower colour polymorphism, pollination modes, breeding system and gene flow in *Anemone coronaria*. *Plants* 9:397. doi: 10.3390/plants9030397

Darwin, C. (1877). *On the Various Contrivances by Which British and Foreign Orchids are Fertilised by Insects*. London: John Murray.

Dellinger, A. S., Artuso, S., Pamperl, S., Michelangeli, F. A., Penneys, D. S., Fernández-Fernández, D. M., et al. (2019). Modularity increases rate of floral evolution and adaptive success for functionally specialized pollination systems. *Commun. Biol.* 2:453. doi: 10.1038/s42003-019-0697-697

Dormont, L., Joffard, N., and Schatz, B. (2019). Intraspecific variation in floral color and odor in orchids. *Int. J. Plant Sci.* 180, 1036–1058. doi: 10.1086/705589

Duffy, K. J., and Johnson, S. D. (2015). Staminal hairs enhance fecundity in the pollen-rewarding self-incompatible lily *Bulbine abyssinica*. *Bot. J. Linn. Soc.* 177, 481–490. doi: 10.1111/boj.12252

Dyer, A. G., and Chittka, L. (2004). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comp. Physiol. A* 190, 105–114. doi: 10.1007/s00359-003-0475-472

Faria, R. T., Santiago, D. C., Saridakis, D. P., Albino, U. B., and Araújo, R. (2002). Preservation of the brazilian orchid *Cattleya walkeriana* Gardner using in vitro propagation. *Crop Breed. Appl. Biot.* 2, 489–492.

Fay, M. F., and Chase, M. W. (2009). Orchid biology: from linnaeus via darwin to the 21st century. *Ann. Bot.* 104, 359–364. doi: 10.1093/aob/mcp190

Fornoni, J., Ordano, M., Pérez-Ishiwara, R., Boege, K., and Domínguez, C. A. (2016). A comparison of floral integration between selfing and outcrossing species: a meta-analysis. *Ann. Bot.* 117, 299–306. doi: 10.1093/aob/mcv166

Gawryszewski, F. M. (2017). Colour vision models: a practical guide, some simulations, and colourvision R package. *bioRxiv* [Preprint], doi: 10.1101/103754

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2002). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.). *Proc. Natl. Acad. Sci. U.S.A.* 98, 6253–6255. doi: 10.1073/pnas.111162598

Heuschen, B., Gumbert, A., and Lunau, K. (2005). A generalised mimicry system involving angiosperm flower colour, pollen and bumblebees' innate colour preferences. *Plant Syst. Evol.* 252, 121–137. doi: 10.1007/s00606-004-0249-245

Hu, A.-Q., Gale, S. W., Liu, Z.-J., Suddee, S., Hsu, T.-C., Fischer, G. A., et al. (2019). Molecular phylogenetics and floral evolution of the *Cirrhopetalum* alliance (Bulbophyllum, Orchidaceae): evolutionary transitions and phylogenetic signal variation. *Mol. Phylogenet. Evol.* 143:106689. doi: 10.1016/J.YMPEV.2019.106689

Jersáková, J., Johnson, S. D., and Kindlmann, P. (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biol. Rev. Camb. Philos. Soc.* 81, 219–235. doi: 10.1017/S1464793105006986

Jiménez-López, F. J., Ortiz, P. L., Talavera, M., and Arista, M. (2019). Selfing maintains flower colour polymorphism in *L. arvensis* despite high inbreeding depression. *bioRxiv* [Preprint], doi: 10.1101/761122

Johnson, S. D., and Schiestl, F. P. (2016). *Floral Mimicry*. Oxford: Oxford University Press.

Juillet, N., and Scopece, G. (2010). Does floral trait variability enhance reproductive success in deceptive orchids? *Perspect. Plant Ecol. Evol. Syst.* 12, 317–322. doi: 10.1016/j.ppees.2010.05.001

Kagawa, K., and Takimoto, G. (2016). Inaccurate color discrimination by pollinators promotes evolution of discrete color polymorphism in food-deceptive flowers. *Am. Nat.* 187, 194–204. doi: 10.1086/684433

Klingenberg, C. P. (2014). Studying morphological integration and modularity at multiple levels: concepts and analysis. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130249. doi: 10.1098/rstb.2013.0249

Koethe, S., Bossems, J., Dyer, A. G., and Lunau, K. (2016). Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*. *J. Comp. Physiol. A* 202, 615–627. doi: 10.1007/s00359-016-1115-y

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmerTest Package: tests in linear mixed effects models. *J. Stat. Softw.* 82:27449. doi: 10.18637/jss.v082.i13

Leonard, A. S., and Papaj, D. R. (2011). "X" marks the spot: the possible benefits of nectar guides to bees and plants. *Funct. Ecol.* 25, 1293–1301. doi: 10.1111/j.1365-2435.2011.01885.x

Li, B. J., Zheng, B. Q., Wang, J. Y., Tsai, W. C., Lu, H. C., Zou, L. H., et al. (2020). New insight into the molecular mechanism of colour differentiation among floral segments in orchids. *Comm. Biol.* 3:89. doi: 10.1038/s42003-020-0821-8

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: flower dummy experiments with bumblebees. *J. Comp. Physiol. A* 166, 827–834. doi: 10.1007/BF00187329

Lunau, K. (1993). Interspecific diversity and uniformity of flower colour patterns as cues for learned discrimination and innate detection of flowers. *Experientia* 49, 1002–1010.

Lunau, K. (1995). Notes on the colour of pollen. *Plant Syst. Evol.* 198, 235–252. doi: 10.1007/BF00984739

Lunau, K. (2000). The ecology and evolution of visual pollen signals. *Plant Syst. Evol.* 222, 89–111. doi: 10.1007/BF00984097

Lunau, K. (2006). Stamens and mimic stamens as components of floral colour patterns. *Bot. Jahrbücher.* 127, 13–41. doi: 10.1127/0006-8152/2006/0127-113

Lunau, K., Konzmann, S., Winter, L., Kamphausen, V., and Ren, Z. X. (2017). Pollen and stamen mimicry: the alpine flora as a case study. *Arthropod. Plant. Interact.* 11, 427–447. doi: 10.1007/s11829-017-9525-9525

Lunau, K., Unseld, K., and Wolter, F. (2009). Visual detection of diminutive floral guides in the bumblebee *Bombus terrestris* and in the honeybee *Apis*

mellifera. *J. Comp. Physiol. A* 195, 1121–1130. doi: 10.1007/s00359-009-0484-x

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 177, 478–489. doi: 10.1007/BF00190178

Maia, R., Gruson, H., Endler, J., and White, T. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1101/427658

Martins, T. R., Jiang, P., and Rausher, M. D. (2017). How petals change their spots: cis-regulatory re-wiring in *Clarkia* (Onagraceae). *New Phytol.* 216, 510–518. doi: 10.1111/nph.14163

Mascó, M., Noy-Meir, I., and Sérsic, A. N. (2004). Geographic variation in flower color patterns within *Calceolaria uniflora* Lam. in Southern Patagonia. *Plant Syst. Evol.* 244, 77–91. doi: 10.1007/s00606-003-0083-81

Medel, R., Botto-Mahan, C., and Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* 84, 1721–1732. doi: 10.1890/01-0688

Mondragón-Palomino, M., and Theißen, G. (2009). Why are orchid flowers so diverse? Reduction of evolutionary constraints by paralogues of class B floral homeotic genes. *Ann. Bot.* 104, 583–594. doi: 10.1093/aob/mcn258

Newman, E., Anderson, B., and Johnson, S. D. (2012). Flower colour adaptation in a mimetic orchid. *Proc. R. Soc. B Biol. Sci.* 279, 2309–2313. doi: 10.1098/rspb.2011.2375

Ordano, M., Fornoni, J., Boege, K., and Domínguez, C. A. (2008). The adaptive value of phenotypic floral integration. *New Phytol.* 179, 1183–1192. doi: 10.1111/j.1469-8137.2008.02523.x

Pansarin, E. R., Bergamo, P. J., Ferraz, L. J. C., Pedro, S. R. M., and Ferreira, A. W. C. (2018). Comparative reproductive biology reveals two distinct pollination strategies in Neotropical twig-epiphyte orchids. *Plant Syst. Evol.* 304, 793–806. doi: 10.1007/s00606-018-1510-7

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A. R., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Pérez, F., Arroyo, M. T. K., and Medel, R. (2007). Phylogenetic analysis of floral integration in *Schizanthus* (Solanaceae): does pollination truly integrate corolla traits? *J. Evol. Biol.* 20, 1730–1738. doi: 10.1111/j.1420-9101.2007.01393.x

R Development Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-785

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* 55, 1269–1282. doi: 10.1111/j.0014-3820.2001.tb00650.x

Schemske, D. W., and Bradshaw, H. D. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci. U.S.A.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Silva, C. I., and Milaneze-Gutierre, M. A. (2005). Aspectos da polinização das orquídeas e o exemplo de Cattleya walkeriana Gardner, nativa dos cerrados brasileiros. *Arqu. Apad.* 9, 16–21.

Silva-Pereira, V., De Camargo Smidt, E., and Leite Borba, E. (2007). Isolation mechanisms between two sympatric *Sophronitis* (Orchidaceae) species endemic to Northeastern Brazil. *Plant Syst. Evol.* 269, 171–182. doi: 10.1007/s00606-007-0583-585

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Smidt, E. C., Silva-Pereira, V., and Borba, E. L. (2006). Reproductive biology of two *Cattleya* (Orchidaceae) species endemic to north-eastern Brazil. *Plant Species Biol.* 21, 85–91. doi: 10.1111/j.1442-1984.2006.00154.x

Telles, F., Klunk, C., Maia, F., Brito, V., and Varassin, I. (2020). Towards a new understanding of the division of labour in heterantherous flowers: the case of *Pterolepis glomerata* (Melastomataceae). *Biol. J. Linn. Soc.* 131, 1–11. doi: 10.1093/biolinnean/blaa107

Telles, F. J., and Rodríguez-Gironés, M. A. (2015). Insect vision models under scrutiny: what bumblebees (*Bombus terrestris terrestris* L.) can still tell us. *Sci. Nat.* 102:4. doi: 10.1007/s00114-014-1256-1251

Thomas, M. M., Rudall, P. J., Ellis, A. G., Savolainen, V., and Glover, B. J. (2009). Development of a complex floral trait: the pollinator-attracting petal spots of the beetle daisy, *Gorteria diffusa* (asteraceae). *Am. J. Bot.* 96, 2184–2196. doi: 10.3732/ajb.0900079

van der Pijl, L., and Dodson, C. H. (1966). *Orchid Flowers: Their Pollination and Evolution*. Coral Gables, FL: University of Miami Press.

Wester, P., and Lunau, K. (2017). Plant-pollinator communication. *Adv. Bot. Res.* 82, 225–257. doi: 10.1016/bs.abr.2016.10.004

Whitney, H. M., Milne, G., Rands, S. A., Vignolini, S., Martin, C., and Glover, B. J. (2013). The influence of pigmentation patterning on bumblebee foraging from flowers of *Antirrhinum majus*. *Naturwissenschaften* 100, 249–256. doi: 10.1007/s00114-013-1020-y

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Wyszecki, G., and Stiles, W. S. (1982). *Color Science: Concepts and Methods, Quantitative Data, and Formulae*, 2nd Edn, New York, NY: John Wiley & Sons.

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Birds Perceive More Intraspecific Color Variation in Bird-Pollinated Than Bee-Pollinated Flowers

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Pollinator-mediated selection is expected to constrain floral color variation within plant populations. Here, we test for patterns of constraint on floral color variation in 38 bee- and/or hummingbird-pollinated plant species from Colorado, United States. We collected reflectance spectra for at least 15 individuals in each of 1–3 populations of each species (total 78 populations) and modeled perceived color variation in both bee and bird visual spaces. We hypothesized that bees would perceive less intraspecific color variation in bee-pollinated species (vs. bird-pollinated species), and reciprocally, birds would perceive less color variation in bird-pollinated species (vs. bee-pollinated species). In keeping with the higher dimensionality of the bird visual system, birds typically perceived much more color variation than bees, regardless of plant pollination system. Contrary to our hypothesis, bees perceived equal color variation within plant species from the two pollination systems, and birds perceived more color variation in species that they pollinate than in bee-pollinated species. We propose hypotheses to account for the results, including reduced long-wavelength sensitivity in bees (vs. birds), and the ideas that potential categorical color vision in birds and larger cognitive capacities of birds (vs. bees) reduces their potential discrimination against floral color variants in species that they pollinate, resulting in less stabilizing selection on color within bird-pollinated vs. bee-pollinated species.

Keywords: plant–pollinator interactions, signaling, reflectance spectra, bee vision, avian vision, natural selection, color polymorphism, color saturation

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INTRODUCTION

Among other traits such as scent and size, flower color is a major signal used by pollinators to identify and choose their host plants (Fenster et al., 2004; Dyer et al., 2012; Schiestl and Johnson, 2013). Because foraging decisions affect visitation, pollination, pollen export and seed set – and thus plant fitness – pollinators can exert selection on flower color (Waser and Price, 1981; Rausher, 2008; Renoult et al., 2013; Caruso et al., 2019). When flower color variants arise via mutation within a plant population, they should be frequently selected against, as pollinators can exhibit positive frequency dependence in their floral color choices (Smithson, 2001; Eckhart et al., 2006). Thus,

a general prediction is that pollinator-driven stabilizing selection should limit intrapopulation variation in floral color (Waser and Price, 1983; Fenster et al., 2004; Rausher, 2008).

Importantly, however, clade-specific color-sensitive receptors and cognitive mechanisms (Renoult et al., 2017) mean that floral color variation is in the eye (and brain) of the beholder. Thus, pollinator-imposed constraints may only be obvious within the bounds of the visual space of the pollinator, and color variation may be less constrained in the visual spaces of non-pollinators (Paine et al., 2019). Central to testing hypotheses about constraints on flower color is the idea of discrimination thresholds within pollinator visual spaces. Within a visual space, greater distance between a pair of colors predicts greater discriminability, but all organisms have thresholds below which discrimination is not possible (e.g., Wyszecki and Stiles, 2000; Dyer and Chittka, 2004; Olsson et al., 2018). For example, within the color-hexagon model of bee vision (Chittka, 1992), bees are typically unable to distinguish colors separated by a Euclidean distance of 0.11 units (e.g., Dyer and Chittka, 2004). Taxon-specific discrimination thresholds allow standardized comparisons of the color variation perceived by different animal taxa (viewing the same set of signals) or perceived by a single animal taxon (viewing different sets of signals).

Using these methods, patterns of floral color variation were recently examined for 34 populations of 14 species of New Mexican bee-pollinated plants, using modeling of visual spaces of bees, birds and humans. For >70% of populations, >95% of pairwise flower–flower comparisons were indistinguishable to bees, consistent with (but not proving) a history of stabilizing selection on flower color mediated by the bee visual system (Paine et al., 2019). Further, these pairs of conspecific flowers were typically visually distinct to humans and birds (non-pollinators of these plants). These findings suggest that human-perceived floral color variation within populations might persist because it is effectively invisible to pollinators. Under these conditions, human-perceived color may evolve neutrally (via drift) or via indirect selection on correlated characters such as drought- or herbivore-resistance, given known pleiotropy between flower pigmentation and these characters (Simms and Bucher, 1996; Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Irwin et al., 2003; Strauss et al., 2004; Vaidya et al., 2018).

Investigations into intraspecific flower color variation are in their infancy (van der Kooi et al., 2019), and specific hypotheses relating perceived intraspecific variation in flower color to the interaction between pollinator visual systems and pollination systems have not yet been developed. While one might be tempted to hypothesize that a pollinator should perceive less color variation within plant species it pollinates, relative to variation perceived by a non-pollinator viewing the same species, this is unlikely to be uniformly true because of differences in overall visual acuity of different animal groups. Dimensionality (the number of receptor types) differs among pollinators. The linear separability of points in any colorspace will generally increase when projected into a higher-dimensional space (Cover, 1965), suggesting that perceived color differences will tend to increase with the number of available input channels. Thus, tetrachromatic birds should typically have finer spectral resolution than trichromatic bees, though taxon-specific variation in receptor sensitivities and post-receptor processing mean that bees can likely achieve finer discrimination than birds in certain regions within the UV-through-green wavelengths (Vorobyev, 1997; Dyer and Chittka, 2004; Caves et al., 2018). These considerations lead us to propose a distinct hypothesis: if pollinator-mediated stabilizing selection has been important in shaping flower color, a pollinator should perceive less color variation within plant species it pollinates, relative to species it does not pollinate (and thus has had no opportunity to shape). In terms of a dataset consisting of flower colors for populations of plant species pollinated by two different pollinator groups, this would manifest as perceived variation being a function of a visual space × pollination system interaction. Here, we test for such a pattern using 78 populations of 38 bee- and/or hummingbird-pollinated plant species from Colorado, United States. We use visual modeling of discrimination thresholds to estimate relative amounts of perceived color variation. Specifically, we hypothesize that bees should perceive less intrapopulation color variation in bee-pollinated (vs. bird-pollinated) plant species; and reciprocally, birds should perceive less intrapopulation color variation in bird-pollinated (vs. bee-pollinated) plant species.

MATERIALS AND METHODS

Study Area

The study was conducted in the Elk Mountains surrounding the Rocky Mountain Biological Laboratory (RMBL) in Gothic, CO, United States (N 38.95807°, W 106.98853°; elev. 2889 m). The area is topographically and biotically diverse (Zorio et al., 2016), with over 1000 species of flowering forbs and shrubs reported from a 10 km radius of RMBL[1]. Bees, flies and hummingbirds (Trochilidae) are the major pollinators in this ecosystem; hummingbirds are the only bird pollinators. Lepidopteran pollinators are present but less common (Miller, 1978; Moldenke and Lincoln, 1979; Campbell et al., 1998). Elevational gradients in flower color in this area have been previously described (Gray et al., 2018).

Study Species and Field Collection

During Summer 2019 (June 1st – August 12th), we opportunistically collected population-level samples from 78 populations of 38 flowering plant species (1–3 populations per species, mean = 2.1). **Table 1** presents the species information. These species generally represented the commonly encountered bee- and bird-pollinated angiosperm forbs and shrubs of the area, but phylogenetic representation was broadened by typically limiting consideration to no more than two species per plant family. However, we targeted bird-pollinated species to increase their representation, since there are fewer bird-pollinated than insect-pollinated species in the area; this resulted in heavier sampling in certain families (e.g., Orobanchaceae; **Table 1**). In total we sampled from 20 families, with a mean of 1.9 and a range of 1–6 species per family (**Table 1**). For all species not readily

[1] soroherbaria.org, downloaded 2020-07-22.

TABLE 1 | Plant species examined in this study and their pollination systems.

Family	Species	Species code	Human hue	Pollination system
Asteraceae	*Balsamorhiza sagittata*	Basa	Yellow to light orange	Bee
	Helianthella quinquenervis	Hequ	Yellow	Bee
Boraginaceae	*Hydrophyllum fendleri*	Hyfe	Pinkish white	Bee
	Mertensia ciliata	Meci	Bright blue to pink or purple	Bee
	Mertensia fusiformis	Mefu	Dark blue to light purple	Bee
Campanulaceae	*Campanula rotundifolia*	Caro	Dark to light purple	Bee
Caprifoliaceae	*Lonicera involucrata*	Loin	Reddish or greenish yellow	Bee/Bird
Fabaceae	*Lathyrus lanszwertii*	Lala	White and pink	Bee
	Lupinus polyphyllus	Lupo	Blue to purple	Bee
	Vicia americana	Viam	Magenta	Bee
Gentianaceae	*Frasera speciosa*	Frsp	White, purple, and green	Bee
Geraniaceae	*Geranium viscosissimum*	Gevi	Pink to white	Bee
Grossulariaceae	*Ribes montigenum*	Rimo	Coral to red	Bee/Fly
Liliaceae	*Erythronium grandiflorum*	Ergr	Yellow	Bee
Linaceae	*Linum lewisii*	Lile	Sky blue to deep blue	Bee/Fly
Montiaceae	*Claytonia lanceolata*	Clla	White and pink	Bee
Onagraceae	*Chamerion angustifolium*	Chan	Pink to fuchsia	Bee
Orobanchaceae	*Castilleja chromosa*	Cach	Deep red	Bird
	Castilleja linariifolia	Cali	Red to orange	Bird
	Castilleja miniata	Cami	Red to orange	Bird
	Castilleja rhexiifolia	Carh	Magenta to lavender	Bird
	Castilleja sulphurea	Casu	Off-white to green	Bee
	Pedicularis bracteosa	Pebr	Greenish white	Bee
Phrymaceae	*Mimulus guttatus*	Migu	Yellow to light orange	Bee
Plantaginaceae	*Penstemon caespitosus*	Peca	Light purple to blue	Bee
	Penstemon rydbergii	Pery	Light purple to blue	Bee
	Penstemon strictus	Pest	Lavender to pinkish	Bee
	Penstemon whippleanus	Pewh	Deep purple to indigo	Bee
Polemoniaceae	*Ipomopsis aggregata*	Ipag	Scarlet	Bird
	Ipomopsis tenuituba	Ipte	Coral red to pink	Bird
Polygonaceae	*Eriogonum umbellatum* var. *aureum*	Erum	Greenish yellow to red	Bee/Fly
Ranunculaceae	*Aquilegia coerulea*	Aqco	White to lavender	Bee/Hawkmoth
	Aquilegia elegantula	Aqel	Red and yellow	Bird
	Delphinium barbeyi	Deba	Light purple to indigo	Bee
	Delphinium nuttallianum	Denu	Purple to blue	Bee/Bird
Rosaceae	*Fragaria virginiana* ssp. *glauca*	Frvi	White	Bee
	Pentaphylloides floribunda	Pefl	Yellow	Bee
Violaceae	*Viola praemorsa*	Vipr	Yellow and brown	Bee/Fly

Colored dots representing the dominant flower hue in human visual space were created by extracting RGB values from a representative pixel from a digital image of each species. Citations for pollination system information are given in ***Supplementary Appendix Table A2****.*

identifiable in the field, one or more voucher specimens were deposited in the RMBL herbarium (specimen list available via soroherbaria.org with Asher K. Smith as collector and "RMBL" as institution).

Population-level samples consisted of an individual flower or inflorescence collected from each of 15 individual plants (1239 total individual plants sampled). For the family Asteraceae, an inflorescence is morphologically integrated to function as a single flower, and therefore we treat their inflorescences as "flowers." Similarly, we collected inflorescences for species for which bracts contribute to showiness and pollinator attraction (e.g., the genus *Castilleja*). We defined populations spatially; we sampled a given species only from locations > 1 km distant or at > 100 m elevation change from other sampling areas and separated by areas where the species was not present.

Pollination System Classifications

To identify the pollinators for each of the 38 plant species, we conducted a literature review, supplemented with local natural history knowledge. Categorizations of the pollination system ("Bee," "Bird," "Bee/Fly," "Bee/Bird," and "Bee/Hawkmoth") for each species and source citations are presented in **Supplementary Appendix Table S2**. Single categorizations (e.g., "Bee") indicate species with a single expected dominant pollinator group, but do not preclude that other pollinator groups contribute in minor roles. Dual categorizations (e.g., "Bee/Fly") denote a mixed

pollination system where either there is no dominant group (e.g., ∼50:50 contributions by two groups), or that there is not enough resolution to identify the dominant group.

Spectrophotometry

We took spectral readings within 12 h of flower collection. For species with multiple human-distinguishable color patches present within a single flower or inflorescence, we chose a patch representing the greatest surface area when considered from the viewpoint of an approaching pollinator. Typically, an individual petal or bract was mounted on cellophane tape and affixed to the bottom of the probe-holder block. The same petal or bract region was used consistently across all individuals within a species (for details on flower preparation for each species, see **Supplementary Appendix Table S3**). Spectrometer readings (spanning 300–700 nm) were taken using an Avantes model 2048 spectrometer, a bifurcated coaxial fiber optic reflectance probe (Avantes FCR-7uv200-2-ME) and an AvaLight-XE xenon light source (Avantes BV, Apeldoorn, Netherlands). Calibration was first made relative to a diffuse white PFTE tile (Avantes WS-2). Integration time was 10 ms. To reduce specular reflectance (Chittka and Kevan, 2005), measurements were taken with the fiber optic probe held at 45° to, and at 8.0 mm from, the flower surface, with the petal tip facing away from the probe. Our sampling design of one spectrum per flower was informed by Dalrymple et al. (2015), which indicated that flower color can be quite precisely estimated with a single measurement.

Spectral Processing and Visual Modeling

We used the R package 'pavo' (Maia et al., 2019) for spectral processing and visual modeling. We first trimmed the spectra to 300–700 nm and set spurious negative reflectance values to zero. We then estimated the subjective perception of floral signals using the receptor-noise limited model for birds (Vorobyev and Osorio, 1998) and the color hexagon model for bees (Chittka, 1992). Each model allows colors to be represented as points in a space delimited by the number and sensitivity of photoreceptors, while accounting for factors such as veiling and incident light, the structure of viewing backgrounds and signals, and more species-specific features of visual processing and perception (Kemp et al., 2015; Maia and White, 2018).

In these color spaces, the distances between points can be interpreted as measures of the subjective difference between colors, with values less than a behaviorally validated 'threshold' of discrimination likely to be indiscriminable to a given viewer. In the receptor-noise limited model for birds, color distances are expressed as weighted Euclidean distances (ΔS), with a value of 1.0 for diurnal birds taken to (conservatively) delimit the threshold below which colors are expected to be indiscriminable under ecologically relevant conditions (reviewed in Wyszecki and Stiles, 2000; Olsson et al., 2018). In the color hexagon for bees, hue is indicated by the radial angle and saturation (spectral purity) is indicated by the distance from the (0,0) origin (Chittka, 1992). Testing of bumblebee and honeybee behavior under laboratory conditions has determined that colors separated by a Euclidean distance of 0.11 'hexagon units' are indiscriminable without aversive differential conditioning, i.e., training with simultaneously presented rewarding and aversive colored stimuli (Dyer and Chittka, 2004; Dyer and Neumeyer, 2005; Dyer, 2006). We used the receptor sensitivities of *Apis mellifera* (Peitsch et al., 1992) as a representative bee pollinator, since the hexagon model is well validated in this species and the sensitivities of photopigments underlying trichromatic vision in the Hymenoptera are highly conserved (Briscoe and Chittka, 2001).

For birds, we used the visual phenotype of an average violet-sensitive (VS) avian viewer for receptor-noise modeling, as the preponderance of evidence suggests that hummingbirds have a VS rather than UVS (ultraviolet-sensitive) system (reviewed in Stoddard et al., 2020). To test the robustness of our results to this assumption, we also modeled birds as UVS; doing so did not qualitatively change any of the patterns or significance levels (results not shown). We specified a relative receptor density of 1:2:2:4 (ultraviolet: short: medium: long wavelength receptors), used a signal-to-noise ratio yielding a Weber fraction of 0.1 and a D65 'standard daylight' illuminant, and assumed that noise is proportional to the Weber fraction and independent of the magnitude of receptor stimulation (Vorobyev and Osorio, 1998).

Statistical Analysis

Background

To explore spectral differences between plants exhibiting different pollination systems, we examined saturation (spectral purity). Bees are expected to have selected for highly saturated colors (Lunau, 1990; Lunau et al., 1996; Rohde et al., 2013). We fit linear mixed-effects models to the data via maximum likelihood using R package 'lme4' (Bates et al., 2015), with saturation as the response variable, and visual system (bee vs. bird), pollination system, and their interaction as predictors. Plant species and population (nested within species) were included as random effects. This analysis focused on only plant species with "Bird" and "Bee" pollination systems (n = 62 populations of 31 species).

Bee and Bird Perception of Intrapopulation Floral Color Variation

To address our focal question, we compared pairwise distances in color space to the relevant discrimination threshold, as delineated above (see also Paine et al., 2019). With 15 samples, there are 15!/(2!(15-2)!) = 105 possible pairwise (flower–flower) comparisons per population. The fraction of these intrapopulation comparisons that are discriminable to a given viewer we call the 'fraction discriminable.' We tabulated comparisons using a custom R script (R Core Team, 2020).

To compare levels of variation perceived by bees vs. birds, we fit linear mixed-effects models to the data via maximum likelihood using R package 'lme4' (Bates et al., 2015). Fraction discriminable was the response variable, with visual system (bee vs. bird), pollination system, and their interaction as predictors. Plant species and population (nested within species) were included as random effects. The main analysis focused on only plant species with "Bird" and "Bee" pollination systems (n = 62 populations of 31 species). To test the robustness of the results to inclusion of the seven species with mixed pollination systems in various ways, separate models were examined where

(1) the two "Bee/Bird" species were included with "Bee," for a total of 33 species; (2) the two "Bee/Bird" species were included with "Bird," for a total of 33 species; and (3) the four "Bee/Fly" and the single "Bee/Hawkmoth" species were both included with "Bee," for a total of 36 species.

To examine whether the amount of floral color variation is correlated across visual spaces, we regressed bird fraction discriminable on bee fraction discriminable. Alternative models with and without two additional predictors, a) pollination system (bee vs. bird) and b) the interaction between pollination system and visual system, were evaluated using AICc. For visualization we used package 'visreg' (Breheny and Burchett, 2017).

RESULTS

Background

Overall, we examined 24 bee-pollinated species, seven bird-pollinated species, and seven species with mixed pollination systems (four "Bee/Fly," one "Bee/Hawkmoth" and two "Bee/Bird"); for reflectance spectra, see **Supplementary Appendix Figure S1**. Colors of bee-pollinated species were more highly saturated than those of bird-pollinated species in bee visual space ($p < 0.0001$), as expected, but birds do not perceive differences in saturation between flowers of the two pollination systems ($p = 0.12$, **Supplementary Appendix Figure S2**).

Bee and Bird Perception of Intrapopulation Floral Color Variation

Across all 38 plant species, mean intrapopulation floral color variation (percent flower pairs discriminable) was 8.6% (range 0–45.7%) within bee visual space, and 56.2% (range 0–92.4%) within bird visual space (**Figure 1**). Across plant populations, bee-perceived and bird-perceived variation was positively correlated (**Supplementary Appendix Figure S3**, $p = 0.0003$, adj. $r^2 = 0.38$).

In the main analysis including only the 24 "Bee" and seven "Bird" species, birds perceive greater variation than bees among flowers of both bee- and bird-pollinated plant species (**Figure 2**; main effect of visual system $\chi^2 = 488.6$, $p < 0.0001$). With regard to our main hypothesis, we did detect the expected visual space × pollination system interaction ($\chi^2 = 30.2$, $p < 0.0001$). However, the patterns ran contrary to the hypothesis: bees did not perceive less color variation in bee-pollinated than bird-pollinated species (instead perceiving equal variation in the two groups, **Figure 2**, contrast t ratio = −0.052, $p = 0.9585$); nor did birds perceive less variation in bird-pollinated than bee-pollinated species (instead, birds perceived more variation in species they pollinate, **Figure 2**, contrast t ratio = −4.695, $p < 0.0001$). As a consequence of this large difference in bird visual space, averaged across visual spaces, bee-pollinated species exhibited overall lower perceived floral color variation than did bird-pollinated species (main effect of pollination system, $\chi^2 = 7.4$, $p = 0.0065$).

The above patterns were qualitatively similar (all effects remained significant and in the same directions) when the two "Bee/Bird" species (*Delphinium nuttallianum*, *Lonicera involucrata*) were included with "Bee" species, when they were

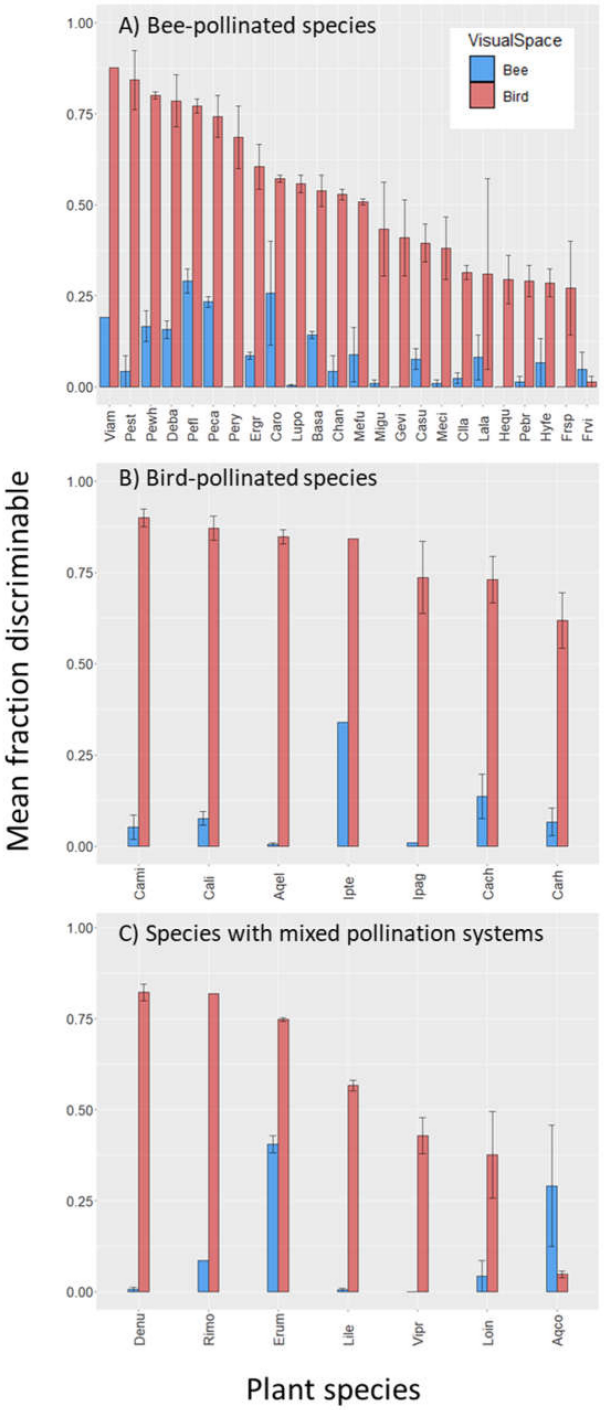


FIGURE 1 | Intrapopulation flower color variation as perceived by bees and birds for 38 species of Rocky Mountain plants. Bars represent the average fraction ± SE (across 1–3 populations per species) of intrapopulation flower–flower comparisons that are discriminable to each viewer. Standard error bars are not present for plant species represented by a single population. Species are presented on the *x*-axis in order of decreasing variation within bird visual space; names associated with the species codes are given in **Table 1**. **(A)** Bee-pollinated species; **(B)** bird-pollinated species; **(C)** species with mixed pollination systems: "Bee/Fly" (Rimo, Lile, Erum, Vipr), "Bee/Hawkmoth" (Acqo), or "Bee/Bird" (Denu, Loin).

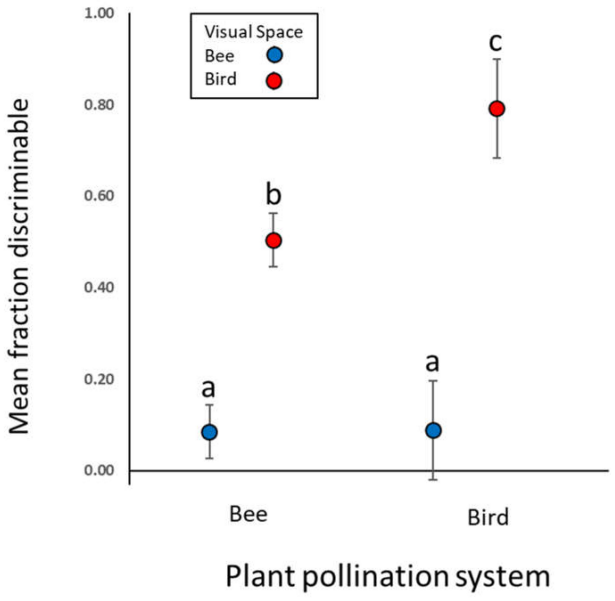


FIGURE 2 | Mean intrapopulation flower color variation in bee and bird visual spaces. Points represent estimated model means (with 95% CI) for the fraction of intrapopulation flower–flower comparisons that are discriminable to each viewer. Data are for 62 populations of 31 Rocky Mountain plant species, grouped by pollination system (24 bee-pollinated and seven bird-pollinated species). Means not sharing a letter differ significantly at $p < 0.05$.

instead included with "Bird" species, or when "Bee/Fly" and "Bee/Hawkmoth" species were both included with "Bee" species (**Supplementary Appendix Table S4**).

DISCUSSION

Our exploration of intrapopulation flower color variation as perceived by bee and bird pollinators found two major patterns. First, birds routinely perceive more intrapopulation variation than do bees; across the plant species examined here, birds were able to distinguish ∼6–9 times more variation than bees (for bee-pollinated and bird-pollinated species, respectively). Second, the evidence is not consistent with the hypothesis that a pollinator should perceive less variation within plant species it pollinates, relative to species it does not pollinate. We estimated that bees perceived equal color variation within populations of bee-pollinated (vs. bird-pollinated) species; further, and in a striking departure from the hypothesis, birds perceived *more* flower color variation within plant species for which they are the dominant pollinator, relative to bee-pollinated plant species.

Bee Perception of Intrapopulation Flower Color Variation

We found that bees typically perceive very little color variation within plant populations that they visit, even for species that exhibit clear variation to humans. These results echo those of Paine et al. (2019), who examined a non-overlapping set of bee-pollinated plant species roughly 450 km to the south of the current study area. However, the inclusion of bird-pollinated species in the current study made it clear that low bee-perceived variation holds for plant species for whom bees play little or no role in pollination. This pattern may arise if the signals of non-bee pollinated species are constrained by their own pollinators (with the shared fundamentals of color vision translating the 'signature' of low-variation to bee visual space), although we note this is unlikely in the current study given the large amount of spectral variation found in populations of bird-pollinated species. Alternatively, the pattern could arise if variation within non-bee pollinated species is biased toward a spectral region to which bees are less sensitive. This scenario is particularly plausible in the current study given that our non-bee pollinators are tetrachromatic birds which possess a richer color-sense that extends into the long-wavelength 'red' region (Chittka, 1992; Hart, 2001; Stoddard et al., 2020). The signals of all seven species classified as bird-pollinated in this study are dominated by long-wavelength reflectance (i.e., they are 'red,' in human-subjective terms; **Table 1**), as consistent with general evidence of partitioning between insect and bird-pollinated flowers along a 'red arm' (Burd et al., 2014). Thus any variation within the 'red arm' of our sampled bird-pollinated species will be relatively difficult for bee viewers to perceive, meshing with a theme in the literature that long-wavelength reflection may be part of a suite of adaptations making it difficult for bees to find and visit bird flowers (Castellanos et al., 2004; Wessinger et al., 2014; Bergamo et al., 2016; see also Chittka and Waser, 1997). Further, with the exception of *Aquilegia elegantula*, none of our seven red bird-pollinated species reflect in the UV (**Supplementary Appendix Figure S1**, panel A). This pattern matches expectations that hummingbird-pollinated red flowers should lack UV reflectance; such flowers are achromatic in bee visual space and difficult for bees to detect against the background, perhaps allowing these floral signals to occupy a 'private niche' for hummingbirds (Lunau et al., 2011). A final general possibility is that low perceived variation may result from forces other than selection by the pollinator group in question. Given connections between the biosynthetic pathways of pigments and other important compounds in plants, flower color can be under indirect selection from many different biotic and abiotic selective agents (Strauss and Whittall, 2006; Caruso et al., 2019).

Bird Perception of Intrapopulation Flower Color Variation

As expected, birds perceived larger amounts of intrapopulation floral color variation than did bees, across the dataset. The simple difference in dimensionality of the two visual spaces will on-balance give tetrachromatic birds finer spectral resolution than trichromatic bees, as generally expected (Cover, 1965; Vorobyev, 1997). In addition, spectral filters, such as oil droplets, are ubiquitous among birds and serve to minimize the overlap in sensitivity between receptor types (Vorobyev, 2003; Hart and Vorobyev, 2005). Such filters enhance discrimination as compared to bee pollinators, whose receptors instead retain

the broad-band sensitivity inherent to visual pigments (Peitsch et al., 1992). Thus our finding that birds likely perceive greater intraspecific floral variation than bees, irrespective of plant pollination system, is consistent with these general differences in their visual systems.

In contrast to our hypothesis that a pollinator should perceive less variation within plant species it pollinates, relative to species it does not pollinate, birds were estimated to perceive *more* variation within bird-pollinated relative to bee-pollinated flowers. This pattern is unlikely to arise simply because (as argued above) birds have an overall richer color-sense than bees. Because birds sample the full visible spectrum relatively efficiently (Vorobyev, 1997), we infer that our bird-pollinated species are indeed more spectrally variable in an absolute sense. We propose several hypotheses to account for this increased variation and the resulting discrepancy between the data and our hypothesis. They share the common theme that birds may not generate as strong selection against color variants as do bees, and thus 'tolerate' higher flower color variation than bees within the plant species that they pollinate.

First, recent research raises the intriguing possibility that categorical color perception may be common among birds both in signaling and non-signaling contexts (Caves et al., 2018; Zipple et al., 2019), and so may shape the functional relevance of apparent signal variation. Categorical perception suggests that (at least some) birds group color stimuli into categories, most likely during post-retinal processing, and canalize a consistent response to those stimuli which share a category despite possessing the low-level sensory apparatus to allow for discrimination (Caves et al., 2018; Zipple et al., 2019). A parallel in human vision is humans' categorical perception of discrete bands in a rainbow, despite the continuously varying wavelengths involved. In examining zebra finch (*Taeniopygia guttata*) vision in the context of mate choice and foraging, boundaries within the orange-red (Caves et al., 2018) and blue-green (Zipple et al., 2019) regions of the spectrum have been documented, such that color pairs on one side of the boundary were less readily distinguished than pairs which spanned it, despite approximately equal color distances between all pairs. For example, in a foraging experiment where food was available under bicolored (but not unicolored) disks, finches were less able to increase foraging success by targeting bicolored disks when the two colors were on one side of the orange-red boundary, than when the two colors spanned the boundary, despite equal discriminability (Caves et al., 2018). As applied to floral signals, then, these results suggest that heightened color variation in bird visual space is not necessarily actionable or functionally relevant, and so (compared to bees) may not as often result in differential fitness among floral color variants.

A related, but more general, possibility is that the larger cognitive capacities of birds (vs. bees) could reduce their potential discrimination against floral color variants in species that they pollinate, resulting in less stabilizing selection on color within the avian visual space than within the bee visual space. In short, the capacities of the avian brain may mean that while birds may perceive greater variation in flower color (as outlined above), they are subsequently able to recognize, classify, and/or remember varying signals as equivalent food resources; thus their foraging decisions may not punish color variants to the extent that bees' might. While bees seem to maintain some level of innate color preference even after accumulating foraging experience (Smithson and Macnair, 1996; Rohde et al., 2013), hummingbirds can be easily trained to switch their color preferences from red to white flowers if the rewards are better (Meléndez-Ackerman et al., 1997). The color signals of bird-pollinated flowers may therefore be (comparatively) less constrained because, unlike bees, such variation is contended with during higher-level processing.

Finally, different pollinating animals emphasize different cues to make their foraging decisions. It may be that color cues are less important to birds than to bees, and thus are subject to less stabilizing selection by the former. Both hummingbirds and bees are known to use olfactory cues to select flowers for visitation (Kessler and Baldwin, 2007; Byers et al., 2014). In a test with *Mimulus* hybrids, pigmentation had a weaker effect on determining hummingbird visitation than bee visitation, with nectar volume (perhaps signaled via scent) serving as a better predictor for hummingbird visitation rates (Schemske and Bradshaw, 1999).

Implications for Floral Evolution

It is possible, though by no means guaranteed, that the apparent high tolerance of flower color variation by bird pollinators means that bird-pollinated lineages could have higher standing genetic variation for flower color (vs. bee-pollinated lineages). Given increasing knowledge about the genetic basis of flower color (e.g., Streisfeld and Rausher, 2009), this hypothesis could be tested. If present, higher standing genetic variation could translate into different rates or trajectories of flower-color evolution or diversification in bird vs. bee-pollinated lineages. Interestingly, diversification rates can be often higher in bird-pollinated than bee-pollinated lineages (e.g., *Aquilegia*, Bastida et al., 2010; Bromeliaceae, Givnish et al., 2014; Gesneriaceae, Serrano-Serrano et al., 2017), but counterexamples exist where the reverse is true (*Penstemon*, Wessinger et al., 2019).

Future Work

Improving our understanding of the relationship between pollinator-mediated selection and floral color variation will require progress on the mechanics of categorical vision (Caves et al., 2018; Zipple et al., 2019). Unfortunately, at this time the categorical boundaries are not mapped with enough precision to apply them to datasets such as ours; for example, the UV region of the spectrum has not been explored for possible boundaries in birds. Further, it is unknown how widespread the phenomenon is among birds beyond zebra finches. Hopefully with progress on mapping boundaries with visual spaces, we could determine if intrapopulation floral color variation tends to span (or not span) category boundaries and thus infer whether selective discrimination between particular floral color variants is even possible.

Further comparative analyses of patterns of floral color variation in different groups are needed to determine how often floral color appears to be constrained within pollinator visual spaces. For example, comparisons of bee- vs. fly-pollinated plant

species should be informative as they would be freer of the noise associated with the vertebrate vs. invertebrate sensory and cognitive differences discussed above.

Finally, it would be beneficial to more explicitly tie current selection to floral color variation. Field studies in wild populations could test whether different pollinator groups impose different amounts of stabilizing selection on flower color via inclusion of quadratic terms in phenotypic selection analyses (Lande and Arnold, 1983). Further, experimental evolution approaches could be used to explicitly document the rate of loss of floral color variation from artificially constructed, high-variance plant populations when exposed to different pollinators. While focused on a different question, the feasibility of this approach has been demonstrated by Gervasi and Schiestl (2017), who found rapid divergence of floral characters in experimental populations of fast cycling *Brassica rapa* exposed to bee vs. hoverfly pollinators.

DATA AVAILABILITY STATEMENT

The dataset generated and analyzed during the current study is available from the Open Science Framework repository at https://doi.org/10.17605/OSF.IO/4B7ES.

AUTHOR CONTRIBUTIONS

KW conceived the idea and led the writing of the manuscript. AS and KW designed the study, and collected plant samples and the spectral data. AS extracted pollinator classifications from the literature. CW collected samples, performed the plant species identifications, and curated the specimens. KW and TW analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.590347/full#supplementary-material

REFERENCES

Bastida, J. M., Alcantara, J. M., Rey, P. J., Vargas, P., and Herrera, C. M. (2010). Extended phylogeny of *Aquilegia*: the biogeographical and ecological patterns of two simultaneous but contrasting radiations. *Plant Syst. Evol.* 284, 171–185. doi: 10.1007/s00606-009-0243-z

Bates, D., Maechler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bergamo, P. J., Rech, A. R., Brito, V. L. G., and Sazima, M. (2016). Flower colour and visitation rates of *Costus arabicus* support the "bee avoidance" hypothesis for red-reflecting hummingbird-pollinated flowers. *Funct. Ecol.* 30, 710–720. doi: 10.1111/1365-2435.12537

Breheny, P., and Burchett, W. (2017). Visualization of regression models using visreg. *R J.* 9, 56–71. doi: 10.32614/RJ-2017-046

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. So. B Biol. Sci.* 281:20132862. doi: 10.1098/rspb.2013.2862

Byers, K. J. R. P., Bradshaw, H. D., and Riffell, J. A. (2014). Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *J. Exp. Biol.* 217, 614–623. doi: 10.1242/jeb.092213

Campbell, D. R., Waser, N. M., and Wolf, P. G. (1998). Pollen transfer by natural hybrids and parental species in an *Ipomopsis* hybrid zone. *Evolution* 52, 1602–1611. doi: 10.1111/j.1558-5646.1998.tb02241.x

Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution* 73, 4–14. doi: 10.1111/evo.13639

Castellanos, M. C., Wilson, P., and Thomson, J. D. (2004). "Anti-bee" and "pro-bird" changes during the evolution of hummingbird pollination in *Penstemon* flowers. *J. Evol. Biol.* 17, 876–885. doi: 10.1111/j.1420-9101.2004.00729.x

Caves, E. M., Green, P. A., Zipple, M. N., Peters, S., Johnsen, S., and Nowicki, S. (2018). Categorical perception of colour signals in a songbird. *Nature* 560, 365–367. doi: 10.1038/s41586-018-0377-7

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 170, 533–543.

Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ON: Enviroquest Ltd), 157–196.

Chittka, L., and Waser, N. M. (1997). Why red flowers are not invisible to bees. *Isr. J. Plant Sci.* 45, 169–183. doi: 10.1080/07929978.1997.10676682

Cover, T. (1965). Geometrical and statistical properties of systems of linear inequalities with applications in pattern recognition. *IEEE Trans. Electron. Comput. EC* 14, 326–344. doi: 10.1109/PGEC.1965.264137

Dalrymple, R. L., Hui, F. K. C., Flores-Moreno, H., Kemp, D. J., and Moles, A. T. (2015). Roses are red, violets are blue - so how much replication should you do? An assessment of variation in the colour of flowers and birds. *Biol. J. Linn. Soc.* 114, 69–81. doi: 10.1111/bij.12402

Dyer, A. G. (2006). Discrimination of flower colours in natural settings by the bumblebee species *Bombus terrestris* (*Hymenoptera*: *Apidae*). *Entomol. Gen.* 28, 257–268.

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., and Chittka, L. (2004). Fine colour discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91, 224–227. doi: 10.1007/s00114-004-0508-x

Dyer, A. G., and Neumeyer, C. (2005). Simultaneous and successive colour discrimination in the honeybee (*Apis mellifera*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 191, 547–557. doi: 10.1007/s00359-005-0622-z

Eckhart, V. M., Rushing, N. S., Hart, G. M., and Hansen, J. D. (2006). Frequency-dependent pollinator foraging in polymorphic *Clarkia xantiana* ssp. xantiana populations: implications for flower colour evolution and pollinator interactions. *Oikos* 112, 412–421.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Gervasi, D. D. L., and Schiestl, F. P. (2017). Real-time divergent evolution in plants driven by pollinators. *Nat. Commun.* 8:14691. doi: 10.1038/ncomms14691

Givnish, T. J., Barfuss, M. H. J., Van Ee, B., Riina, R., Schulte, K., and Horres, R. (2014). Adaptive radiation, correlated and contingent evolution, and net species diversification in Bromeliaceae. *Mol. Phylogenet. Evol.* 71, 55–78. doi: 10.1016/j.ympev.2013.10.010

Gray, M., Stansberry, M. J., Lynn, J. S., Williams, C. F., White, T. E., and Whitney, K. D. (2018). Consistent shifts in pollinator-relevant floral coloration along rocky mountain elevation gradients. *J. Ecol.* 106, 1910–1924. doi: 10.1111/1365-2745.12948

Hart, N. S. (2001). The visual ecology of avian photoreceptors. *Prog. Retin. Eye Res.* 20, 675–703. doi: 10.1016/S1350-9462(01)00009-X

Hart, N. S., and Vorobyev, M. (2005). Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 191, 381–392. doi: 10.1007/s00359-004-0595-3

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., and Dyer, A. G. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Natural.* 185, 705–724. doi: 10.1086/681021

Kessler, D., and Baldwin, I. T. (2007). Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *Plant J.* 49, 840–854. doi: 10.1111/j.1365-313X.2006.02995.x

Lande, R., and Arnold, S. (1983). The measurement of selection on correlated characters. *Evolution* 37, 1210–1226. doi: 10.2307/2408842

Lunau, K. (1990). Color saturation triggers innate reactions to flower signals - flower dummy experiments with bumblebees. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 166, 827–834.

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 178, 477–489.

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Maia, R., and White, T. E. (2018). Comparing colors using visual models. *Behav. Ecol.* 29, 649–659. doi: 10.1093/beheco/ary017

Meléndez-Ackerman, E., Campbell, D. R., and Waser, N. M. (1997). Hummingbird behavior and mechanisms of selection on flower color in Ipomopsis. *Ecology* 78, 2532–2541.

Miller, R. B. (1978). The pollination ecology of *Aquilegia elegantula* and *A. caerulea* (*Ranunculaceae*) in Colorado. *Am. J. Bot.* 65, 406–414.

Moldenke, A. R., and Lincoln, P. G. (1979). Pollination ecology in montane Colorado: a community analysis. *Phytologia* 42, 349–379.

Olsson, P., Lind, O., and Kelber, A. (2018). Chromatic and achromatic vision: parameter choice and limitations for reliable model predictions. *Behav. Ecol.* 29, 273–282. doi: 10.1093/beheco/arx133

Paine, K. C., White, T. E., and Whitney, K. D. (2019). Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints? *Evol. Ecol.* 33, 461–479. doi: 10.1007/s10682-019-09991-2

Peitsch, D., Fietz, A., Hertel, H., Desouza, J., Ventura, D., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 170, 23–40. doi: 10.1007/BF00190398

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Kelber, A., and Schaefer, H. M. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Renoult, J. P., Thomann, M., Schaefer, H. M., and Cheptou, P.-O. (2013). Selection on quantitative colour variation in *Centaurea cyanus*: the role of the pollinator's visual system. *J. Evol. Biol.* 26, 2415–2427. doi: 10.1111/jeb.12234

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* 55, 1269–1282.

Schemske, D. W., and Bradshaw, H. D. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci. U.S.A.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Serrano-Serrano, M. L., Rolland, J., Clark, J. L., Salamin, N., and Perret, M. (2017). Hummingbird pollination and the diversification of angiosperms: an old and successful association in Gesneriaceae. *Proc. R. Soc. B -Biol. Sci.* 284:20162816. doi: 10.1098/rspb.2016.2816

Simms, E. L., and Bucher, M. A. (1996). Pleiotropic effects of flower-color intensity on herbivore performance on *Ipomoea purpurea*. *Evolution* 50, 957–963. doi: 10.2307/2410871

Smithson, A. (2001). "Pollinator preference, frequency dependence, and floral evolution," in *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*, eds L. Chittka and J. D. Thomson (Cambridge: Cambridge University Press), 237–258.

Smithson, A., and Macnair, M. R. (1996). Frequency-dependent selection by pollinators: mechanisms and consequences with regard to behaviour of bumblebees *Bombus terrestris* (L) (*Hymenoptera*: *Apidae*). *J. Evol. Biol.* 9, 571–588. doi: 10.1046/j.1420-9101.1996.9050571.x

Stoddard, M., Eyster, H., Hogan, B., Morris, D., Soucy, E., and Inouye, D. W. (2020). Wild hummingbirds discriminate nonspectral colors. *Proce. Natl. Acad. Sci. U.S.A.* 117, 15112–15122.

Strauss, S. Y., Irwin, R. E., and Lambrix, V. M. (2004). Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *J. Ecol.* 92, 132–141. doi: 10.1111/j.1365-2745.2004.00843.x

Strauss, S. Y., and Whittall, J. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Streisfeld, M. A., and Rausher, M. D. (2009). Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species. *New Phytol.* 183, 751–763. doi: 10.1111/j.1469-8137.2009.02929.x

Vaidya, P., McDurmon, A., Mattoon, E., Keefe, M., Carley, L., and Lee, C.-R. (2018). Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (*Boechera stricta*). *New Phytol.* 218, 380–392. doi: 10.1111/nph.14998

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

Vorobyev, M. (1997). "Costs and benefits of increasing the dimensionality of colour vision system," in *Biophysics of Photoreception: Molecular and Phototransductive Events*, ed. C. Taddei-Ferretti (Singapore: World Scientific Publishing), 280–289.

Vorobyev, M. (2003). Coloured oil droplets enhance colour discrimination. *Proc. R. Soc. B Biol. Sci.* 270, 1255–1261. doi: 10.1098/rspb.2003.2381

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Waser, N. M., and Price, M. V. (1981). Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* 35, 376–390. doi: 10.1111/j.1558-5646.1981.tb04896.x

Waser, N. M., and Price, M. V. (1983). Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302, 422–424. doi: 10.1038/302422a0

Wessinger, C. A., Hileman, L. C., and Rausher, M. D. (2014). Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130349. doi: 10.1098/rstb.2013.0349

Wessinger, C. A., Rausher, M. D., and Hilema, L. C. (2019). Adaptation to hummingbird pollination is associated with reduced diversification in *Penstemon*. *Evol. Lett.* 3, 521–533. doi: 10.1002/evl3.130

Wyszecki, G., and Stiles, W. S. (2000). *Color science: Concepts and Methods, Quantitative Data, and Formulae*. New York: John Wiley & Sons.

Zipple, M. N., Caves, E. M., Green, P. A., Peters, S., Johnsen, S., and Nowicki, S. (2019). Categorical colour perception occurs in both signalling and non-signalling colour ranges in a songbird. *Proc. R. Soc. B Biol. Sci.* 286:20190524. doi: 10.1098/rspb.2019.0524

Zorio, S. D., Williams, C. F., and Aho, K. A. (2016). Sixty-five years of change in montane plant communities in western Colorado, USA. *Arctic Antarctic Alp. Res.* 48, 703–722. doi: 10.1657/AAAR0016-011

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Floral Color Variation in *Drosera cistiflora* Is Associated With Switches in Beetle Pollinator Assemblages

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Floral color shifts are thought to be one of the most common evolutionary transitions in plants, and pollinators are often proposed as important selective agents driving these transitions. However, shifts in flower color can also be related to neutral genetic processes or pleiotropy linked with selection via other biotic agents or abiotic factors. Here we ask whether abiotic factors or pollinators provide the best explanation for divergence in flower color among populations of the sundew *Drosera cistiflora* s.l. (Droseraceae). This species complex in the Greater Cape Floristic Region contains at least five distinctive floral color forms. Abiotic factors do not appear to play a significant role in color determination, as the forms are not specific to a single soil or vegetation type, sometimes co-occur in the same habitat, and maintain their color traits in common-garden and soil switching experiments. Instead, we found strong associations between flower color and the composition of pollinator assemblages which are dominated by hopliine scarab beetles. Pollinator assemblages show geographical structuring, both within and among color forms. This makes it difficult to dissect the roles of geography versus floral traits in explaining pollinator assemblages, but strong pollinator partitioning among color forms at sites where they are sympatric indicates that pollinators may select strongly on color. These results suggest that beetle pollinators are a significant factor in the evolution of *D. cistiflora* s.l. flower color.

Keywords: ecotypes, geographical races, local adaptation, hopliine beetle, phenotypic plasticity, pollination, pollen rewards, speciation

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INTRODUCTION

Pollinator-mediated selection on floral traits is considered a major driver of floral divergence (Grant and Grant, 1965; Fenster et al., 2004; Harder and Johnson, 2009; Schiestl and Johnson, 2013; Gervasi and Schiestl, 2017). It follows that spatial variation in pollinator assemblages may generate divergent selective pressures among plant populations, as proposed by the Grant-Stebbins pollinator-shift model (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006; Kay and Sargent, 2009; Van der Niet et al., 2014).

Convergent evolution of flower color is well documented for guilds of plants pollinated by the same pollinator functional group (Fenster et al., 2004) and this provides one line of evidence

for pollinator-mediated selection on floral color evolution. Another is divergence in flower color among closely related plants that have pollinators differing in color preference. Indeed, there is now some compelling evidence for this process, albeit limited to few studies (Meléndez-Ackerman, 1997; Schemske and BradshawJr., 1999; Irwin and Strauss, 2005; Hopkins and Rausher, 2012; Streinzer et al., 2019). Array experiments involving *Mimulus* species (Phrymaceae) and their hybrids, for example, have confirmed flower color discrimination by both hummingbirds and bees, and further linked pollinator preferences to specific gene regions associated with petal pigmentation and nectar volume (Schemske and BradshawJr., 1999). By using arrays of both model and reciprocally translocated flowers, Newman et al. (2012) attributed geographical variations in flower color of the orchid *Disa ferruginea* to pollinator color preferences shaped by positive associative conditioning in local communities. New evidence also suggests that elevational segregation of flower color in *Anemone pavonina* (Ranunculaceae) may be linked to the relative importance for pollination of glaphyrid beetles versus other insects along environmental gradients (Streinzer et al., 2019). In addition, numerous experimental studies with flower-visiting animals have revealed strong innate color preferences as well as ability to develop color preferences through associative conditioning (Lunau et al., 1996; Weiss, 1997; Pohl et al., 2008; Ings et al., 2009).

Although these studies suggest that pollinators can be very important selective agents in floral color transitions, and even the main driving force behind these shifts, no study has demonstrated unequivocally that different assemblages of pollinators are the agents of selection behind geographical floral color shifts (Rausher, 2008). Several other hypotheses for color shifts have been proposed (Narbona et al., 2018), including nonadaptive evolution as a result of genetic drift (Wright, 1943) or the (indirect) consequence of pleiotropic effects of genes relating to physiological or vegetative adaptation to environmental conditions (Rausher and Fry, 1993; Levin and Brack, 1995; Warren and Mackenzie, 2001; Armbruster, 2002; Strauss and Whittall, 2006; Arista et al., 2013). For example, the anthocyanin pigment may confer abiotic stress tolerance to seedlings, and, since its presence in seedlings can also determine flower color (Bowman, 1987; Strauss and Whittall, 2006), floral color transitions may be maintained through selection on seedling traits. In *Acer* (Sapindaceae) red and purple flowers evolved in lineages where anthocyanins are present in leaves, while pale-green or yellow flowers evolved in lineages without anthocyanins in leaves (Armbruster, 2002). Alternatively, flower color genes may have pleiotropic effects on water use physiology, which in turn may result in geographical structuring of flower colors, as proposed for *Linanthus parryae* (Polemoniaceae) by Schemske and Bierzychudek (2001). Floral color divergence may also be maintained through the pleiotropic effects of flower color genes on herbivory (Irwin et al., 2003) and seed predation (Carlson and Holsinger, 2010, 2013). Lastly, floral color shifts may represent plastic responses to differing edaphic conditions such as geographical soil mosaics, as determined by variation in physical and/or chemical components of the soil (Ito et al., 2009). A well-known example is *Hydrangea macrophylla* (Hydrangeaceae), which can vary from blue to pink depending on soil pH (Ito et al., 2009; Schreiber et al., 2010). It is clear, therefore, that studies of flower color evolution should include an integrated consideration of biotic and abiotic factors that may explain transitions between flower colors (Herrera, 1996; Galen, 1999a,b; Ellis and Johnson, 2009).

Drosera cistiflora s.l. is a perennial, pollinator-dependent, insectivorous plant species complex endemic to the Fynbos Biome in the Greater Cape Floristic Region of South Africa. Plants produce inflorescences with 1–5 large flowers (30–55 mm in width) which open consecutively (usually only one flower is presented at a time) and are hermaphroditic, actinomorphic and bowl-shaped. There is a spectacular diversity of floral color forms in the complex, ranging through pink, purple, red, white and yellow (von Witt, 2019). Though currently recognized as a single species, *D. cistiflora* may be best characterized as a species complex consisting of numerous forms at different stages of divergence. Some floral color forms can co-exist without producing visible intermediates and may represent emerging lineages with near-identical morphology (von Witt, 2019). The purple, red, and yellow flower colors are discrete, but there can be a gradient between pink- and white-flowered forms in some populations, while other populations are entirely white- or pink-flowered. The flowers of *D. cistiflora* s.l. are pollen-rewarding and devoid of nectar and discernible scent, making them particularly suitable for studies on the role of flower color in influencing pollinator attraction. Evidence suggests that *D. cistiflora* s.l. is pollinated primarily by hopliine beetles [Coleoptera: Scarabaeidae: Hopliini] (Goldblatt et al., 1998; Anderson, 2010; von Witt, 2019). All floral color forms are dependent on pollinators for seed production, which is moderately to highly pollen-limited (von Witt, 2019; von Witt et al., 2020).

This study explores two alternative hypotheses: i) that floral color variation in *D. cistiflora* s.l. is correlated with abiotic factors such as soils or ii) that it is associated with pollinator assemblages. If floral color divergence is a plastic response to soils, or if it is an evolved response to differences in components of the physical environment, then we predict that populations with the same flower color should occur in similar soil and vegetation types. In addition, plastic responses to soils should also be characterized by changes in flower color when plants are grown in different soils. If floral color variation is driven by pollinator-mediated selection, then we expect that populations with the same flower color should have similar suites of insect pollinators, and that populations with different flower colors should have different pollinator assemblages.

MATERIALS AND METHODS

Study Sites

Study sites were chosen to represent extant populations of *Drosera cistiflora* s.l. (**Supplementary Table S1**). We studied 16 populations representing the following flower colors

(n = populations): pink (4), purple (2), red (4), white (3), and yellow (3).

Geographical Distribution of Floral Color Forms

To determine whether there is any geographical pattern in flower color distribution, we mapped all records of historical *D. cistiflora* s.l. populations in South Africa according to flower color wherever this was documented in the collector's notes. Records were obtained from specimens housed in the Compton and Bolus Herbaria; data collected by the Custodians of Rare and Endangered Wildflowers (CREW) program, and field excursions carried out in our personal capacity. These were mapped using ARCVIEW GIS 3.2. Voucher specimens are housed in the Compton Herbarium.

We determined the spectral reflectance over the UV–visible range (300–700 nm) of a sample from 5 to 16 petals, each from a separate plant, in populations of each color form. Populations sampled (see **Supplementary Table S1** for site details) were Darling 6 and 7 (pink-flowered form); Darling 2 and Durbanville (purple-flowered form); Darling 1, 2 and 3, and Darling-Yzerfontein (red-flowered form); Betty's Bay, and Darling 4 and 5 (white-flowered form), and Piketberg 1, 2, and 3 (yellow-flowered form). We used an Ocean Optics (Dunedin, FL, United States) S2000 spectrophotometer and Ocean Optics DT-mini deuterium tungsten halogen light source (200–1,100 nm). We took reflectance readings from the outer section of the petals by placing the fiber optic reflection probe (UV/VIS 400 μm) at a 45° angle from the surface of the petal.

Soil Types and Vegetation

To investigate the potential for edaphic specialization in *D. cistiflora* s.l. floral color forms, we determined the underlying geology and soil structure of populations of each floral color form by overlaying the 1:250,000 geology layer (2010) from the Western Cape Department of Agriculture onto point locality data of *D. cistiflora* s.l. populations with recorded flower colors (**Supplementary Table S2**).

A common-garden and soil switching experiment was conducted to test whether differences in soil chemistry and altered environmental conditions influenced expression of flower color. We allocated plants in bud, from each of the floral forms to different potting treatments: Plants were either planted in their soil of origin, or in soils taken from the sites of each of the other color forms, or in a soil from a site without *D. cistiflora*. We used three plants per treatment per color form, making up a sample of 18 plants per color form and 90 plants in total. These were then moved to a common site and observed for changes in flower color (**Supplementary Figure S1**). Controls consisted of plants potted in their original soil. Plants and soils were obtained from Darling 7 (granite and granodiorite soils supporting the pink-flowered form); Darling 2 (loam and sandy loam soils; purple-flowered form); Darling 3 (loam and sandy loam soils; red-flowered form); Darling 4 (granite and granodiorite soils; white-flowered form), and Piketberg 1 (grit and greywacke soils; yellow-flowered form). For the soil treatment from a site without *D. cistiflora* s.l., we used clay soils collected from The Towers Farm, Darling.

Plants were potted at the beginning of the flowering season in 2010 and observations made until flowering ceased at the end of each season, up until 2013. All experimental plants were kept in common environmental conditions at all times, and these common conditions were altered when the experiment was transferred from Darling to the Kirstenbosch National Botanical Garden Collections Nursery greenhouse of the South African National Biodiversity Institute after the flowering season in 2010. The plants were thus exposed to changes in soil temperature, light and moisture availability when they were removed from their native sites and also during the course of the experiment. Flower color was allocated to the pink, purple, red, white and yellow categories by the human eye.

Considering that plant communities share similar abiotic environmental conditions, community classification may act as a surrogate for overall abiotic factors to be compared between *D. cistiflora* s.l. populations. Thus, to assess whether populations of the same *D. cistiflora* s.l. floral color form are associated with similar plant communities, we established the vegetation type of each population by overlaying the 1:250,000 vegetation layer (Mucina and Rutherford, 2006) onto point locality data for extant populations of all *D. cistiflora* s.l. floral color forms.

Pollinator Assemblages

To determine whether the flower color of a *D. cistiflora* s.l. population was associated with the pollinator community, flower visitors were observed in 16 populations of five floral color forms in 2009 and 2010 on sunny, windless days during periods of peak pollinator activity: 09h30–15h00. Each site measured approximately 50 × 50 m. Where possible, 250 flowers were randomly checked for the presence of pollinators. Where fewer than 250 flowers were present, all flowers in the population were checked (**Supplementary Table S1**) and observation numbers standardized across populations. The abundance and kinds of flower visitors were noted and at least one voucher specimen of each visitor was captured for identification. Insects that came into contact with floral reproductive parts were considered to be pollinators. Individual insects were killed by freezing and kept in separate vials to avoid pollen contamination. All insects were identified to family or subfamily, and genus and species where possible.

The potential importance of each insect species as a *D. cistiflora* s.l. pollen vector was calculated as the product of its relative abundance as a visitor to *D. cistiflora* s.l. flowers within a population and the average number of *D. cistiflora* s.l. pollen grains that it carried. Pollen grains were counted under a dissecting microscope for 1–12 (median = 5) individuals (in some cases fewer than five individuals were captured) of all observed insect visitor species and classified as *D. cistiflora* s.l. pollen or "other." Pollen grains were identified by comparison with a reference set of microscope slide preparations of pollen grains made from *D. cistiflora* s.l. and co-occurring plants at all study sites. Relative pollinator importance (RPI) was calculated as the percentage contribution of each pollinator to the overall pollinator importance for each *D. cistiflora* s.l. floral color form.

If pollinators discriminate among floral color forms or if pollinators are geographically associated with particular flower colors, then we would expect that populations of the same flower color will be visited by similar assemblages of insects. This was tested with non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis index of pollinator species composition for *D. cistiflora* s.l. floral color forms. Three analyses were performed, one using relative pollinator abundances in each *D. cistiflora* s.l. population, another using an estimate of the importance of each pollinator species (as the product of relative abundance and average pollen loads) and another restricted to the relative abundance data for hopliine scarab beetles. Permutation tests comparing pollinator assemblages among flower colors were applied using ANOSIM implemented in PRIMER 6.1.15.

The relationship between geographical proximity of *D. cistiflora* s.l. floral color forms and the similarity of pollinator communities was assessed to determine whether pollinator assemblages were geographically structured and whether similarities in pollinator assemblages within or among floral color forms could reflect spatial proximity of populations. Pairwise distances between each population were calculated using ARCVIEW GIS 3.2, and a geographical distance matrix was produced. Mantel tests implemented in POPTOOLS (Hood, 1994) were used to assess the relationship between pairwise geographical distance and the Bray-Curtis index of the pollinator community composition of each population pair. Three separate analyses were performed, using data for: i) all populations, ii) populations with different flower colors, and iii) populations with the same flower color.

RESULTS

Geographical Distribution of Floral Color Forms

Locality data were found for 168 *Drosera cistiflora* s.l. populations of known flower color in South Africa (**Figure 1**). The range of these sites spanned 685 km from west to east and 390 km from north to south. The majority of sites comprised either pink (108 sites: 64.3% of all sites) or white (41 sites: 24.4%) floral color forms. These two forms are widespread throughout the entire range of the species complex. Populations designated here as pink may show a continuum spanning pink to white, but populations designated as white consisted only of white-flowered individuals. Small clusters of three additional floral color forms are found in the central part of the range (**Figure 1**). Six yellow-flowered populations (3.6% of total sites) were found within 40 km of each other. To the south of these, nine red-flowered populations (5.3% of total sites) occurred within 83 km of each other, and further south, four purple-flowered populations (2.4% of total sites) were found within 38.5 km of each other. Two of the red-flowered populations co-occurred with purple-flowered

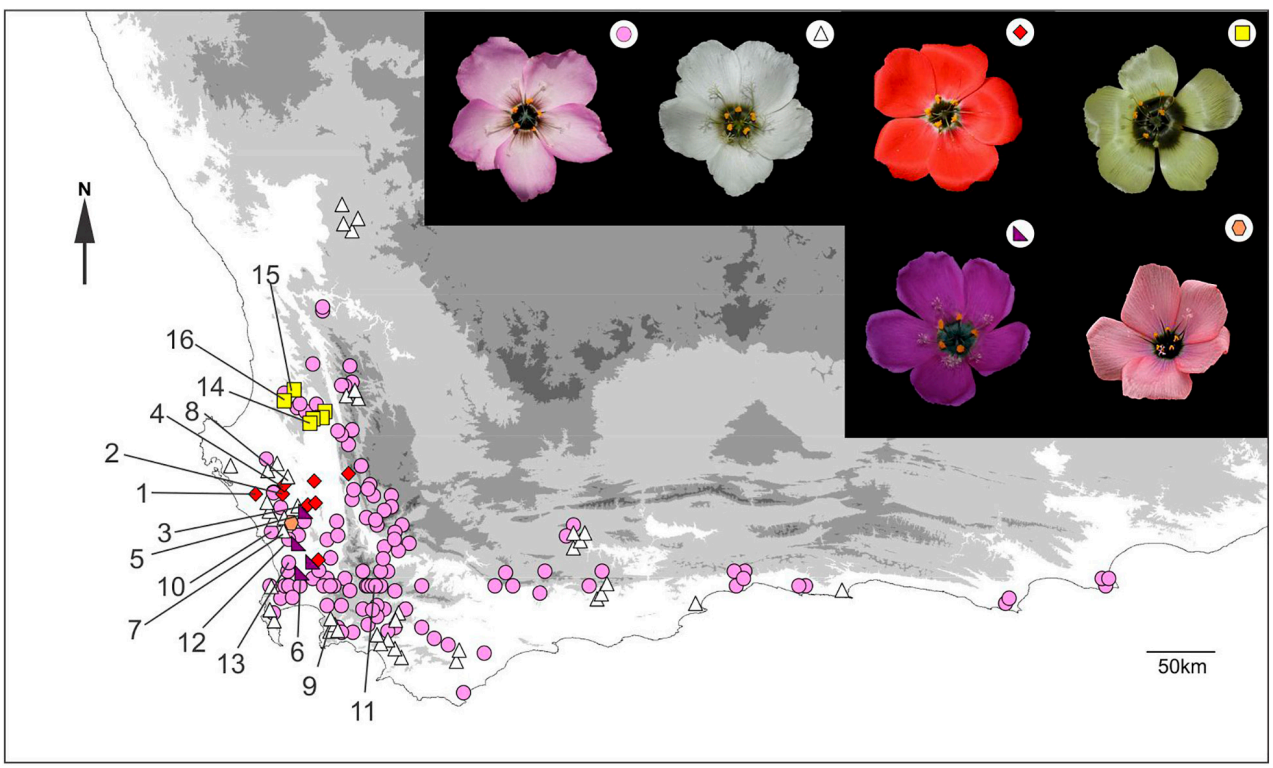


FIGURE 1 | The geographical distribution of all known extant and extinct populations of *Drosera cistiflora* s.l. where corolla color data has been recorded. Recorded flower colors (see symbols) in decreasing order of frequency are pink (circles), white (triangles), red (diamonds), yellow (squares), purple (right-angled triangles), and salmon pink (hexagons). Populations studied (**Supplementary Table S1**) are labeled numerically. Photo of salmon pink form by Rob Maharajh.

individuals and another one co-occurred with white-flowered individuals. Purple-flowered populations also co-occurred with pink- or white-flowered populations at two sites. Apart from pink and white forms which sometimes showed a continuum, the different flower colors appeared to be discrete with no intermediates observed in zones where the distribution ranges of different forms overlapped.

Measurements of reflectance spectra showed that UV light is not reflected by the upper petal surfaces of any of the floral color forms. Spectra of pink and white forms were not readily distinguishable, but spectra of red, yellow, and purple forms were discrete (**Figure 2**).

Soil Types and Vegetation

Populations of *D. cistiflora* s.l. occur on at least 21 soil types. No floral color forms were edaphic endemics as all occurred on two or more soil types. Pink- and white-flowered forms were found on the most diverse range of soil types, reflecting the much larger distribution range of these two floral color forms. There was also considerable overlap of soil types between populations with different flower colors. For example, pink, purple, red, and white flowers could all be found on loam soils and pink, purple and white flowers could all be found on sandy soils (**Supplementary Tables S2, S3**).

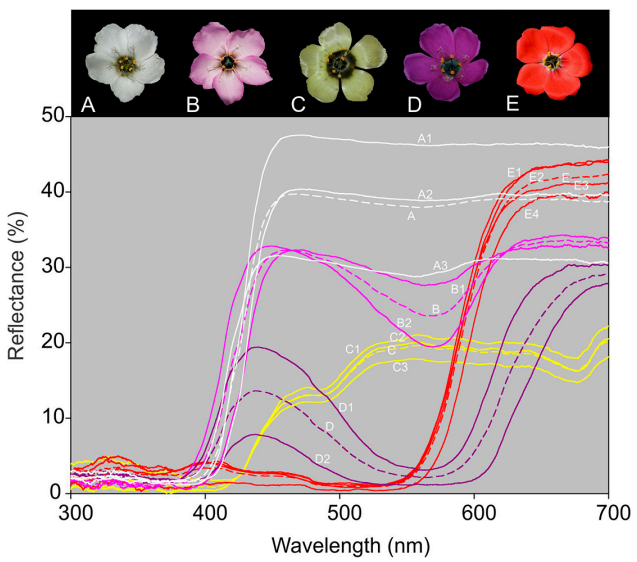


FIGURE 2 | Spectrophotometer readings over the UV–visible range (300–700 nm) for the outer region of the petals of all five *Drosera cistiflora* s.l. floral color forms. Readings were obtained from 5 to 16 flowers from two populations of each of the pink **(B)** and purple **(D)**; three populations of white **(A)** and yellow **(C)**, and four of red **(E)** *D. cistiflora* s.l. floral color forms. Populations sampled comprised: Darling 5 (A1), Darling 4 (A2), and Betty's Bay (A3) [white-flowered form]; Darling 7 (B1) and Darling 6 (B2) [pink-flowered form]; Piketberg 1 (C1), Piketberg 2 (C2), and Piketberg 3 (C3) [yellow-flowered form]; Darling 2 (D1) and Durbanville (D2) [purple-flowered form], and Darling 2 (E1), Darling 1 (E2), Darling 3 (E3), and Darling-Yzerfontein (E4) [red-flowered form]. Average readings for each floral color form are distinguished by dashed lines.

No change in floral color expression was apparent for any of the 90 potted plants (18 per color form) with switched substrates and altered environmental conditions when they were examined in 2010, 2012, and 2013.

Each *D. cistiflora* s.l. floral color form was found in more than one vegetation type and most of these vegetation types supported more than one floral color form (**Supplementary Table S4**). For example, Atlantis Sand Fynbos and Swartland Granite Renosterveld support pink, purple, red and white floral color forms; Swartland Shale Renosterveld supports pink, purple and yellow floral color forms, and both red- and white-flowered forms also occur in Hopefield Sand Fynbos. The distribution of *D. cistiflora* s.l. flower colors was therefore not tightly linked with any specific plant communities.

Pollinator Assemblages

We recorded a total of 1,169 individual insects as visitors to flowers of *D. cistiflora* s.l. (**Supplementary Table S5**). A total of 27 insect pollinator species from 11 families were observed in *D. cistiflora* s.l. flowers in 2009 and 2010 (summary in **Table 1**, details in **Supplementary Table S5**). The overwhelming majority (74%) of insect species recorded were beetles and most (44%) of these were hopliine scarabs (**Table 1**, **Supplementary Table S5**).

Hopliine beetles (Coleoptera: Scarabaeidae: Hopliini) were evidently the primary pollinators of purple, red, white and yellow *D. cistiflora* s.l. floral color forms (**Table 1**, **Figure 3**, and **Supplementary Table S5**), with relative importance (RPI) per floral color form in descending order of magnitude as follows: red (99.7%), white (91.7%), purple (80.4%), yellow (71.8%), and pink (31.8%). Species assemblages of hopliine beetles differed largely between colors. Non-florivorous beetles of the family Meloidae were also important pollinators of pink-flowered forms (RPI: 50.5%), and soft-winged flower beetles (Melyridae) were of importance in pink-, purple-, and yellow-flowered forms, with RPI of 13.4, 19.2, and 21.8%, respectively. A florivorous lunate blister beetle *Hycleus lunatus* (Coleoptera: Meloidae: Meloinae: Mylabrini) was observed to consume flower parts (**Supplementary Figure S2**) and was excluded from the lists of potential pollinators. A species of hopliine beetle, *Lepisia rupicola*, had particularly high relative importance in red- (RPI: 88.7%), white- (RPI: 87.1%), and pink-flowered (RPI: 29.6%) populations, but was absent from yellow-flowered populations (**Table 1**, **Supplementary Table S5**). All *D. cistiflora* s.l. insect visitors were found to be polylectic (viz. not specific to *D. cistiflora* s.l.) and carried pollen from other plant species in the local environment.

There was an overall relationship between flower color and pollinator community (**Figure 4**). In particular, red- and yellow-flowered populations formed distinct clusters on the basis of pollinator composition. Red-flowered populations clustered strongly and had a significantly different pollinating fauna to yellow- ($R = 1.00$, $p = 0.03$) and pink-flowered populations ($R = 0.54$, $p = 0.03$) and a marginally non-significant difference to white-flowered populations ($R = 0.39$, $p = 0.06$), while the fauna in yellow-flowered populations was significantly different to that in

TABLE 1 | Relative importance (RPI) values (%) of insect pollinators observed visiting each floral color form of *Drosera cistiflora* s.l. in 2009 and 2010.

		***Drosera cistiflora* s.l. floral color form**				
Order and family	**Species**	**Pink**	**Purple**	**Red**	**White**	**Yellow**
Coleoptera						
Scarabaeidae: Hopliini	*Anisochelus inornatus*					16.5
	Anisonyx sp.				4.4	
	Anisonyx cf. ursus	2.3	2.0		0.19	
	Chasme decora			10.6		
	Chasme sp.			0.07		
	Heterochelus sp.					1.2
	Lepisia rupicola	29.6	15.2	**88.7**	**87.1**	
	Lepithrix sp.					**46.8**
	Omocrates sp.		**63.2**			
	Peritrichia sp. 1					6.2
	Peritrichia sp. 2					0.99
	Platychelus lupinus			0.28		
Scarabaeidae	sp. 1	1.3				0.28
Chrysomelidae	sp. 1	0.3		0.01	0.02	
Meloidae	sp. 1	**50.5**			4.5	
Melyridae	sp. 1	0.05				
	sp. 2	1.02			0.81	21.8
	sp. 3	13.4	19.2		2.5	2.6
Tenebrionidae	sp. 1			0.02		
	sp. 2	0.66			0.02	
Hymenoptera						
Megachilidae	sp. 1	0.50			0.32	
Diptera						
Muscidae	sp. 1	0.23				
Tabanidae	sp. 1			0.27		
	sp. 2					3.4
Ceratopogonidae	sp. 1	0.03	0.09	0.002	0.04	0.054
Empididae	sp. 1	0.04				
Hemiptera						
Lyganidae	sp. 1				0.121	

*Pollinator importance was calculated as the product of abundance in D. cistiflora s.l. flowers and average D. cistiflora s.l. pollen loads (**Supplementary Table S5**). Relative importance was calculated as the percentage contribution of each pollinator to the overall pollinator importance in each D. cistiflora s.l. floral color form. The pollinator group with the highest RPI value for each floral color form is indicated in bold type.*

pink-flowered populations ($R = 0.46$, $p = 0.03$). Populations of pink and white floral color forms had more variable pollinator compositions and did not form discrete clusters. Given that there were only two purple-flowered populations, there was not enough statistical power to compare this form to the others.

Clustering weighted by relative pollinator importance did not strongly alter the associations between pollinator assemblage and flower color (global $R = 0.48$, $p = 0.02$, stress value = 0.09), so that red- and yellow-flowered populations still formed discrete groups (**Supplementary Figure S3**). Yellow-flowered populations were significantly different from those of red- ($R = 1.00$, $p = 0.03$) and pink-flowered ($R = 0.51$, $p = 0.03$) populations. The difference between red-flowered and pink-flowered populations was marginally non-significant ($R = 0.46$, $p = 0.057$) and all other pairwise combinations were non-significant.

In the NMDS analysis restricted to hopliine scarab beetle assemblages, there was again strong clustering of red- and yellow-flowered populations (**Supplementary Figure S4**), but red-flowered populations were not clearly separable from white- and pink-flowered populations and the only significant differences were between red- and yellow-flowered populations ($R = 1$, $P = 0.029$) and between yellow- and pink-flowered populations ($R = 0.52$, $P = 0.029$).

There was a significant negative relationship between pairwise geographical proximity of *D. cistiflora* s.l. populations and the pairwise similarity of the pollinating fauna $r_m = -0.474$, $P < 0.0001$ (**Figure 5A**), indicating that nearby populations shared similar pollinators while geographically distant populations had more dissimilar pollinating fauna compositions. This was also the case for pairs of populations with different flower colors ($r_m = -0.417$, $P < 0.0001$, **Figure 5B**) and those with the same flower color ($r_m = -0.547$, $P < 0.0001$, **Figure 5C**).

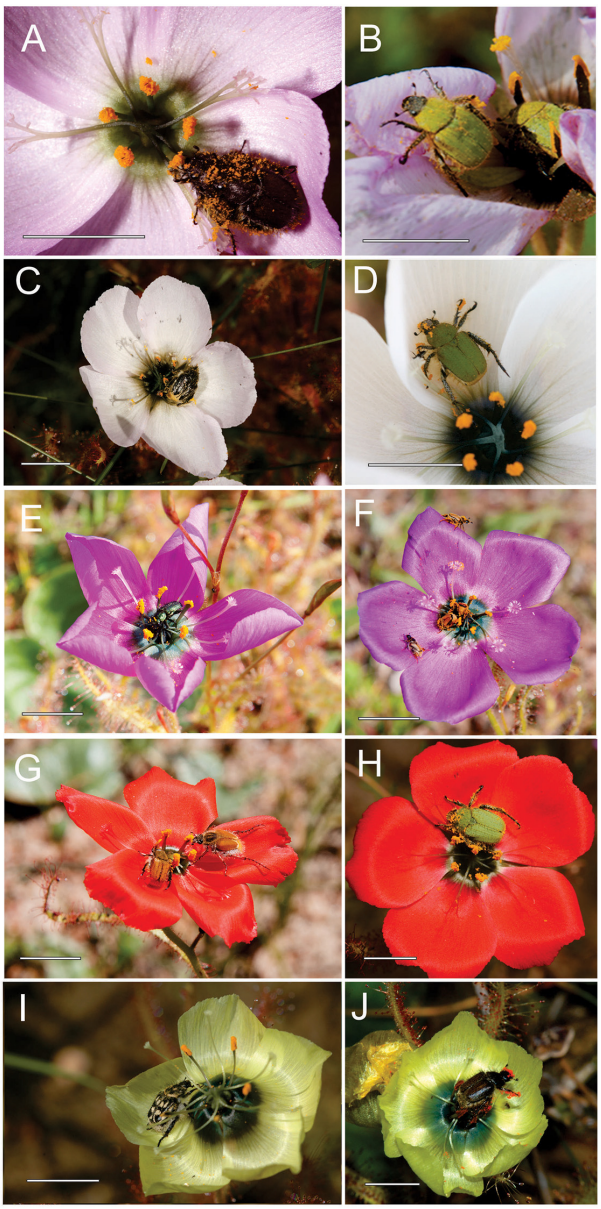


FIGURE 3 | Hopliine beetle (Coleoptera: Scarabaeidae: Hopliini) **(A–D,F–J)** and melyrid beetle (Coleoptera: Melyridae) **(E)** pollinators on the five floral color forms of *Drosera cistiflora* s.l. *Lepisia rupicola* spec. **(B,D,H)** emerged as an important pollinator of pink- **(B)**, white- **(D)** and red-flowered **(H)** forms. *Omocrates* sp. **(F)** was abundant in purple-flowered forms and *Chasme decora* (**G**, left) was only observed on red flowers. *Peritrichia* sp. **(I)** and *Heterochelus* sp. **(J)** are shown visiting yellow flowers. Photo **(J)** by Kim Steiner. Scale bars = 10 mm.

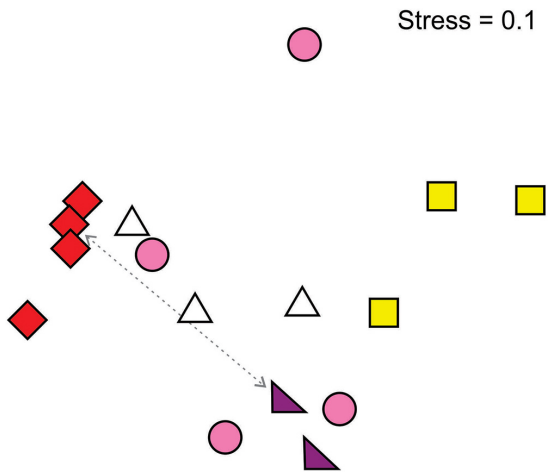


FIGURE 4 | Multidimensional scaling (Bray-Curtis similarity index) plot of pollinator assemblages according to the flower color of *Drosera cistiflora* s.l. populations. Populations that are close together share similar pollinator communities while those that are far apart have different pollinator communities. Symbols differentiate *D. cistiflora* s.l. flower colors (see **Figure 1** for a key). Two populations that are sympatric are indicated by a dotted line.

DISCUSSION

Floral color variation in the *D. cistiflora* complex is associated with switches in beetle pollinator assemblages dominated by hopliine scarab beetles. It is particularly notable that hopliine scarabs, along with glaphyrid scarabs in the Mediterranean, are among the few insect groups to include species that are strongly attracted to red flowers (Picker and Midgley, 1996; Johnson and Midgley, 2001; Van Kleunen et al., 2007; Streinzer et al., 2019). However, unlike glaphyrid scarabs that show consistent preferences for red and do not seem to clearly discriminate other colors, such as white, from the background foliage (Martinez-Harms et al., 2012; Streinzer et al., 2019), hopliine scarabs show a wide range of color preferences that vary markedly among individual species (Picker and Midgley, 1996; Steiner, 1998b; Johnson and Midgley, 2001; Van Kleunen et al., 2007; von Witt, 2019). Hopliine scarabs are thought to be responsible for a syndrome of dark-centered and bowl-shaped flowers with colors ranging from white through to red that have evolved in many different lineages in the Cape Floristic Region (Goldblatt et al., 1998; Van Kleunen et al., 2007). *Drosera cistiflora* provides an unusual case of a species complex where the divergence among floral color forms appears to have been driven by the foraging preferences of hopliine scarabs (von Witt, 2019).

We found no evidence suggesting that underlying soil or habitat type is associated with the color of *D. cistiflora* s.l. flowers. In fact, several sites (**Supplementary Tables S1, S2**) have more than one floral color form, occasionally growing intermingled. It is also clear that the natural distribution of each floral color form transcends many different soil types. In view of the absence of edaphic and vegetation type endemism in all *D. cistiflora* s.l. floral color forms, and given that vegetation types may serve as a proxy for multiple abiotic factors such as soil chemistry, temperature, light and moisture availability, flower color in *D. cistiflora* s.l. does not appear to be a manifestation of physiological responses to components of the physical environment. Furthermore, individuals transplanted across soil types also retain their original color over many flowering seasons, suggesting that color variation is not a phenotypically plastic trait. The transplant experiment does not provide conclusive evidence

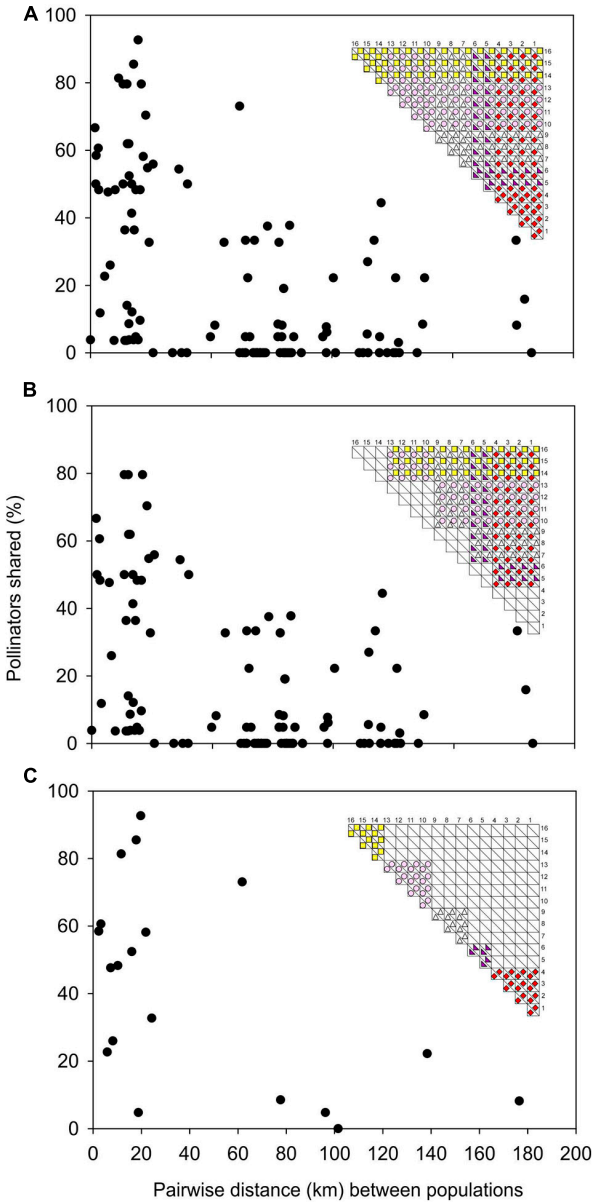


FIGURE 5 | Relations between pairwise geographical distances and pairwise Bray-Curtis similarities of flower visitor assemblages in *Drosera cistiflora* s.l. populations, for all populations **(A)**, populations with different flower colors only **(B)**, and populations with the same flower color only **(C)**. Inset matrices show populations 1–16 and flower colors for which correlations are plotted. Each dot represents a population pair.

for this on its own, as the plants were not grown from seed (germination of the seeds is extremely difficult to achieve in cultivation). However, when seen in the context of co-existence of some color forms and a lack of overall association between soils and color, the evidence indicates that flower color is not simply a plastic response to soil chemistry.

Flower color in the genus *Drosera* globally and in South Africa is generally pink or white and this is also the case for most populations in the *D. cistiflora* complex (von Witt, 2019). The relatively terminal position of the species complex in the phylogeny of *Drosera* (Rivadavia et al., 2003) suggests that novel colors in the complex, such as red and yellow, are more recently derived modifications. The red- and yellow-flowered forms are specialized for pollination by hopliine scarab beetles and are also strongly diverged from one another in terms of their pollination niches. We suspect that these represent the evolution of novel pigment pathways (rather than loss of function transitions which tend to occur much more commonly – Rausher, 2008). Transitions involving loss of function can easily occur under very weak natural selection, or can even occur as a result of genetic drift when selection on color is weak or absent. However, it can be expected that the evolution of novel pigment pathways would have to occur under conditions of very strong directional selection. In this case the independent evolution of red and yellow colors in numerous other unrelated South African angiosperms pollinated by hopliine beetles (Steiner et al., 1987; Goldblatt et al., 1998; Johnson et al., 2004) provides a pattern of convergent evolution which is strongly suggestive of adaptive function. Another convergent feature found in c. 75% of plant species pollinated by hopliine scarabs is a dark floral center (Goldblatt et al., 1998; Johnson and Midgley, 2001). It has been suggested that these dark centers could represent mimicry of potential mating partners (Steiner, 1998a). There is evidence that dark floral centers increase the frequency of landings by hopliine beetles (Van Kleunen et al., 2007), but the strongest effect on their alighting behavior is the overall flower color (Johnson and Midgley, 2001). We frequently observed mating of hopliine scarabs on flowers of *D. cistiflora*, s.l. but the color of the dark center of the flowers does not usually correspond closely to the elytra of the beetles, which range in color from light green through to dark brown-black across species (**Figure 3**).

Closely related populations are expected to share traits through common descent and should also be geographically close to one another. We detected strong associations between geographic distance and pollinator community composition, suggesting that geographical proximity of similar color forms alone may explain associations between flower color and pollinator community composition. However, if geography was the only explanation for pollinator assemblages, different color forms within a site would be expected to have the same pollinator assemblages. This is not the case because at sites with more than one flower color we find strong pollinator partitioning among color forms. For example, the Darling 2 site has co-flowering purple and red forms, where the pollinator assemblage of the red form is more similar to that of relatively geographically distant red populations than the co-occurring purple-flowered plants (**Figure 4**). At this site, two hopliine beetle pollinators (*Lepisia rupicola* and *Chasme decora*) made up more than 95% of all insect visits to red flowers but made up only 3.8% of visits to purple flowers. In contrast, the most important visitors to purple flowers at this site were the hopliine beetle *Omocrates* sp. and melyrid beetles which were never observed on the red flowers. This pollinator partitioning also suggests a potentially strong role played by pollinators in reproductive isolation of emerging lineages (Liu and Huang, 2013). All *D. cistiflora* s.l. floral color forms, whether they occurred together at the same site, or apart at

different sites, had overlapping flowering phenologies. However, while this suggests that pollinators select among flowers on the basis of color, it does not demonstrate that geographical color variation is locally adaptive. We have obtained evidence for local adaptation in a separate study in which arrays of different color forms of *D. cistiflora* s.l. were presented at each site (von Witt, 2019). This confirmed that there is strong discrimination by beetles among color forms and an overall preference of beetles for the local color forms.

A fundamental assumption of the Grant-Stebbins model of pollinator-driven diversification is that pollinator communities differ geographically (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006, 2010; Van der Niet et al., 2014). If the most effective pollinators in these different communities differ in their flower color preferences, we would expect a geographical selection mosaic that would result in the evolution of geographical differences in color forms. Since we sampled pollinators only on *Drosera* flowers, we have no independent assessment of pollinator distributions and the museum records of these insects are too sparse to create reliable distribution maps. This is a very common problem in studies that attempt to test the Grant-Stebbins model of diversification driven by geographical mosaics of pollinator availability (Johnson, 2006). However, plots of pollinator sharing by populations in relation to geographical distance (**Figure 5**) show that there is some degree of geographical structuring of pollinator assemblages that is independent of floral color forms. While this is not conclusive evidence for ultimate geographical structure in pollinator availability (as opposed to the proximate effects of floral traits on pollinator assemblages), it is consistent with one of the predictions of the Grant-Stebbins model.

Floral color shifts in *D. cistiflora* s.l. appear to represent adaptations to entire community compositions of pollinators and not specific species, since pollinator assemblages differed overall but also had many overlapping components. This would be consistent with the assertion of Gómez et al. (2008, 2014) that floral phenotypes of generalist plants may diversify across the range of a species in response to suites of pollinators (many of which may also be generalist). Similarly, findings of generalized pollination and spatial variation in visitor assemblage in *Calochortus* (Liliaceae) suggested that lineages may have been moving through a spatiotemporal mosaic of pollinators over evolutionary time (Dilley and Mesler, 2000). Here the authors surmised that color patterns, among other floral traits, have diverged through the historical accumulation of floral modifications that have been selected for by the suites of pollinators to which they appeal. Given that hopliine scarabs display highly variable color preferences among species (Picker and Midgley, 1996; Steiner, 1998b; Johnson and Midgley, 2001; Van Kleunen et al., 2007), spatiotemporal variation in pollinator assemblages that include hopliine beetles is a highly plausible explanation for the evolution of the floral color polymorphism in *D. cistiflora* s.l.

Although floral color forms of *D. cistiflora* s.l. clearly transcend soil and habitat types, we could expect steep environmental gradients to shape pollinator assemblages if insects use specific soils as nesting sites, or use soil-specific larval host plants. Indeed, this appears to be the case for hopliine beetles, which display remarkably high levels of species turnover across very short geographic distances, often corresponding to changes in vegetation and soil (Colville et al., 2002; Colville, 2009). As a result, many hopliine beetles are endemic to very narrow habitats (Colville, 2009). Thus the ultimate reasons for geographical structure in assemblages of these beetles may relate to their general habitat requirements. Indeed, shifts in pollination systems have frequently been associated with parallel shifts in soil types (Patterson and Givnish, 2004; Goldblatt and Manning, 2006; Van der Niet et al., 2006), and it is plausible that future examination of *D. cistiflora* s.l. pollinator biogeography may actually find pollinator assemblages to be determined in part by edaphic and/or other abiotic factors. As experiments show that beetle pollinators of *D. cistiflora* s.l. discriminate strongly among the color forms (von Witt, 2019), there is potential for floral colour divergence to proceed as "consequent radiation" (sensu Patterson and Givnish, 2004) via an indirect association of plants with the soils and/or other physical components of the environment supporting their pollinators.

CONCLUSION

Our results show a pattern linking pollinator communities and flower color, and as such the most compelling explanation for floral color divergence in *D. cistiflora* s.l. is that it has been pollinator-driven. Hopliine beetles show strong color preferences when selecting flowers (Johnson and Midgley, 2001; Van Kleunen et al., 2007; von Witt, 2019). Studies have revealed at least three photoreceptor types in certain hopliine beetle species (Arnold, 2010), and this is consistent with findings of local color preferences of pollinators in the *D. cistiflora* complex (von Witt, 2019).

In addition to pollinator color choice experiments, further work should include study of *D. cistiflora* s.l. pigment biosynthetic pathways and their pleiotropic potential, and pleiotropic effects of non-pollinator biotic agents such as herbivores, pollen thieves and seed predators. Detailed molecular studies of *D. cistiflora* s.l. plant populations may ultimately isolate genetic differences between floral color forms and determine whether these are associated with pollinator shifts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors participated in the design of the study. SDJ and CvW contributed equally as joint first authors. This manuscript is based on a Ph.D. thesis by CvW who collected the data and wrote up the results under the supervision of SDJ and BA. SDJ led the

process of preparing the manuscript for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.606259/full#supplementary-material

REFERENCES

Anderson, B. (2010). Did *Drosera* evolve long scapes to stop their pollinators from being eaten? *Ann. Bot.* 106, 653–657. doi: 10.1093/aob/mcq155

Arista, M., Talavera, M., Berjano, R., and Luis Ortiz, P. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Armbruster, W. S. (2002). Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *J. Evol. Biol.* 15, 468–486. doi: 10.1046/j.1420-9101.2002.00399.x

Arnold, S. E. J. (2010). *Flowers Through Insect Eyes: The Contribution of Pollinator Vision to the Evolution of Flower Colour*. PhD thesis. Queen Mary: University of London.

Bowman, R. N. (1987). Cryptic self-incompatibity and the breeding system of *Clarkia unguiculata* (Onagraceae). *Am. J. Bot.* 74, 471–476. doi: 10.1002/j.1537-2197.1987.tb08667.x

Carlson, J. E., and Holsinger, K. E. (2010). Natural selection on inflorescence color polymorphisms in wild *Protea* populations: the role of pollinators, seed predators and inter-trait correlations. *Am. J. Bot.* 97, 934–944. doi: 10.3732/ajb.0900348

Carlson, J. E., and Holsinger, K. E. (2013). Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171, 905–919. doi: 10.1007/s00442-012-2453-2

Colville, J. (2009). *Understanding the Evolutionary Radiation of the Megadiverse Monkey Beetle Fauna (Scarabaeidae: Hopliini) of South Africa*. PhD thesis. Cape Town: University of Cape Town.

Colville, J., Picker, M. D., and Cowling, R. M. (2002). Species turnover of monkey beetles (Scarabaeidae: Hopliini) along environmental and disturbance gradients in the Namaqualand region of the succulent Karoo, South Africa. *Biodivers. Conserv.* 11, 243–264.

Dilley, J. D., and Mesler, M. R. (2000). The radiation of *Calochortus*: generalist flowers moving through a mosaic of potential pollinators. *Oikos* 89, 209–222. doi: 10.1034/j.1600-0706.2000.890201.x

Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.3732/ajb.0800222

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403.

Galen, C. (1999a). Flowers and enemies: predation by nectar-thieving ants in relation to variation in floral form of an alpine wildflower, *Polemonium viscosum*. *Oikos* 85, 426–434. doi: 10.2307/3546692

Galen, C. (1999b). Why do flowers vary? The functional ecology of variation in flower size and form within natural plant populations. *BioScience* 49, 631–640. doi: 10.2307/1313439

Gervasi, D. D. L., and Schiestl, F. P. (2017). Real-time divergent evolution in plants driven by pollinators. *Nat. Commun.* 8:14691. doi: 10.1038/ncomms14691

Goldblatt, P., Bernhardt, P., and Manning, J. C. (1998). Pollination of petaloid geophytes by monkey beetles (Scarabaeidae: Rutelinae: Hopliini) in southern Africa. *Ann. Missouri Bot. Gar.* 85, 215–230. doi: 10.2307/2992006

Goldblatt, P., and Manning, J. C. (2006). Radiation of pollination systems in the iridaceae of sub-Saharan Africa. *Ann. Bot.* 97, 317–344. doi: 10.1093/aob/mcj040

Gómez, J. M., Bosch, J., Perfectti, F., Fernandez, J. D., Abdelaziz, M., and Camacho, J. P. M. (2008). Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators. *Proc. R. Soc. B Biol. Sci.* 275, 2241–2249. doi: 10.1098/rspb.2008.0512

Gómez, M. J., Perfectti, F., Abdelaziz, M., Lorite, J., Munoz-Pajares, A. J., and Valverde, J. (2014). Evolution of pollination niches in a generalist plant clade. *New Phytol.* 205, 440–453. doi: 10.1111/nph.13016

Grant, V., and Grant, K. A. (1965). *Flower Pollination in the Phlox Family*. New York, NY: Columbia University Press.

Harder, L. D., and Johnson, S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytol.* 183, 530–545. doi: 10.1111/j.1469-8137.2009.02914.x

Herrera, C. M. (1996). "Floral traits and plant adaptation to insect pollinators: a devil's advocate approach," in *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants*, eds D. G. Lloyd and S. C. H. Barrett (New York, NY: Chapman & Hall), 65–87. doi: 10.1007/978-1-4613-1165-2_3

Hood, M. E. (1994). *Poptools Version 2.6.2*. Canberra, CBR: CSIRO.

Hopkins, R., and Rausher, M. D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science* 335, 1090–1092. doi: 10.1126/science.1215198

Ings, T. C., Raine, N. E., and Chittka, L. (2009). A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 63, 1207–1218. doi: 10.1007/s00265-009-0731-8

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am. Natural.* 165, 225–237. doi: 10.2307/3473147

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-9658(2003)084[1733:TROHIT]2.0.CO;2

Ito, D., Shinkai, Y., Kato, Y., Kondo, T., and Yoshida, K. (2009). Chemical studies on different color development in blue- and red-colored sepal cells of *Hydrangea macrophylla*. *Biosci. Biotechnol. Biochem.* 73, 1054–1059. doi: 10.1271/bbb.80831

Johnson, S. D. (2006). "Pollinator-driven speciation in plants," in *The Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 295–310.

Johnson, S. D. (2010). The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 499–516. doi: 10.1098/rstb.2009.0243

Johnson, S. D., Collin, C. L., Wissman, H. J., Halvarsson, E., and Agren, J. (2004). Factors contributing to variation in seed production among remnant

populations of the endangered daisy *Gerbera aurantiaca*. *Biotropica* 36, 148–155. doi: 10.1646/q1569

Johnson, S. D., and Midgley, J. F. (2001). Pollination by monkey beetles (Scarabaeidae: Hopliini): do color and dark centers of flowers influence alighting behavior? *Environ. Entomol.* 30, 861–868. doi: 10.1603/0046-225x-30.5.861

Kay, K. M., and Sargent, R. D. (2009). The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annu. Rev. Ecol. Evol. Syst.* 40, 637–656. doi: 10.1146/annurev.ecolsys.110308.120310

Levin, D. A., and Brack, E. T. (1995). Natural selection against white petals in *Phlox*. *Evolution* 49, 1017–1022. doi: 10.2307/2410423

Liu, C.-Q., and Huang, S.-Q. (2013). Floral divergence, pollinator partitioning and the spatiotemporal pattern of plant-pollinator interactions in three sympatric *Adenophora* species. *Oecologia* 173, 1411–1423. doi: 10.1007/s00442-013-2723-7

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 178, 477–489.

Martinez-Harms, J., Vorobyev, M., Schorn, J., Shmida, A., Keasar, T., Homberg, U., et al. (2012). Evidence of red sensitive photoreceptors in *Pygopleurus israelitus* (Glaphyridae: Coleoptera) and its implications for beetle pollination in the southeast Mediterranean. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 198, 451–463. doi: 10.1007/s00359-012-0722-5

Meléndez-Ackerman, E. J. (1997). Patterns of color and nectar variation across an *Ipomopsis* (Polemoniaceae) hybrid zone. *Am. J. Bot.* 84, 41–47. doi: 10.2307/2445881

Mucina, L., and Rutherford, M. C. (eds). (2006). *The vegetation of South Africa, Lesotho and Swaziland. Strelitzia 19*. South African National Biodiversity Institute, Pretoria.

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Newman, E., Anderson, B., and Johnson, S. D. (2012). Flower colour adaptation in a mimetic orchid. *Proc. R. Soc. B Biol. Sci.* 279, 2309–2313. doi: 10.1098/rspb.2011.2375

Patterson, T. B., and Givnish, T. J. (2004). Geographic cohesion, chromosomal evolution, parallel adaptive radiations, and consequent floral adaptations in *Calochortus* (Calochortaceae): evidence from a cpDNA phylogeny. *New Phytol.* 161, 253–264. doi: 10.1046/j.1469-8137.2003.00951.x

Picker, M. D., and Midgley, J. J. (1996). Pollination by monkey beetles (Coleoptera: Scarabaeidae: Hopliini): flower and colour preferences. *Afr. Entomol.* 4, 7–14.

Pohl, M., Watolla, T., and Lunau, K. (2008). Anther-mimicking floral guides exploit a conflict between innate preference and learning in bumblebees (Bombus terrestris). *Behav. Ecol. Sociobiol.* 63, 295–302. doi: 10.1007/s00265-008-0661-x

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Rausher, M. D., and Fry, J. D. (1993). Effects of a locus affecting floral pigmentation in *Ipomoea purpurea* on female fitness components. *Genetics* 134, 1237–1247.

Rivadavia, F., Kondo, K., Kato, M., and Hasebe, M. (2003). Phylogeny of the sundews, *Drosera* (Droseraceae), based on chloroplast rbcL and nuclear 18S ribosomal DNA sequences. *Am. J. Bot.* 90, 123–130. doi: 10.3732/ajb.90.1.123

Schemske, D. W., and Bierzychudek, P. (2001). Evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. *Evolution* 55, 1269–1282. doi: 10.1554/0014-3820(2001)055[1269:peofci]2.0.co;2

Schemske, D. W., and Bradshaw, H. D. Jr. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci. U.S.A.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Schreiber, H. D., Swink, A. M., and Godsey, T. D. (2010). The chemical mechanism for Al3+ complexing with delphinidin: a model for the bluing of hydrangea sepals. *J. Inorgan. Biochem.* 104, 732–739. doi: 10.1016/j.jinorgbio.2010.03.006

Stebbins, G. L. (1970). Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annu. Rev. Ecol. Syst.* 1, 307–326. doi: 10.1146/annurev.es.01.110170.001515

Steiner, A., Paul, R., and Gemperlein, R. (1987). Retinal receptor types in *Aglais urticae* and *Pieris brassicae* (Lepidoptera), revealed by analysis of the electroretinogram obtained with Fourier interferometric stimulation (FIS). *J. Comp. Physiol. A* 160, 247–258. doi: 10.1007/bf00609730

Steiner, K. E. (1998a). Beetle pollination of peacock moraeas (Iridaceae) in South Africa. *Plant Syst. Evol.* 209, 47–65. doi: 10.1007/bf00991523

Steiner, K. E. (1998b). The evolution of beetle pollination in a South African orchid. *Am. J. Bot.* 85, 1180–1193. doi: 10.2307/2446351

Strauss, S. Y., and Whittall, J. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Streinzer, M., Roth, N., Paulus, H. F., and Spaethe, J. (2019). Color preference and spatial distribution of glaphyrid beetles suggest a key role in the maintenance of the color polymorphism in the peacock anemone (*Anemone pavonina*, Ranunculaceae) in Northern Greece. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 205, 735–743. doi: 10.1007/s00359-019-01360-2

Van der Niet, T., Johnson, S. D., and Linder, H. P. (2006). Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution* 60, 1596–1601. doi: 10.1554/05-705.1

Van der Niet, T., Peakall, R., and Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Ann. Bot.* 113, 199–211. doi: 10.1093/aob/mct290

Van Kleunen, M., Nanni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle marks and flower colour on visitation by monkey beetles (Hopliini) in the greater cape floral region. South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

von Witt, C. G. (2019). *Pollination and Geographical Divergence in Flower Colour of the Drosera cistiflora Species Complex*. PhD thesis. Pietermaritzburg: University of KwaZulu-Natal.

von Witt, C. G., Anderson, B., Durbach, I. N., and Johnson, S. D. (2020). Breeding systems of floral colour forms in the *Drosera cistiflora* species complex. *Plant Biol.* 22, 992–1001.

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Weiss, M. R. (1997). Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Anim. Behav.* 53, 1043–1053. doi: 10.1006/anbe.1996.0357

Wright, S. (1943). An analysis of local variability of flower color in *Linanthus parryae*. *Genetics* 28, 139–156.

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Changing How Biologists View Flowers—Color as a Perception Not a Trait

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Studying flower color evolution can be challenging as it may require several different areas of expertise, ranging from botany and ecology through to understanding color sensing of insects and thus how they perceive flower signals. Whilst studies often view plant-pollinator interactions from the plant's perspective, there is growing evidence from psychophysics studies that pollinators have their own complex decision making processes depending on their perception of color, viewing conditions and individual experience. Mimicry of rewarding flowers by orchids is a fascinating system for studying the pollinator decision making process, as rewarding model flowering plants and mimics can be clearly characterized. Here, we focus on a system where the rewardless orchid *Eulophia zeyheriana* mimics the floral color of *Wahlenbergia cuspidata* (Campanulaceae) to attract its pollinator species, a halictid bee. Using recently developed psychophysics principles, we explore whether the color perception of an insect observer encountering variable model and mimic flower color signals can help explain why species with non-rewarding flowers can exist in nature. Our approach involves the use of color discrimination functions rather than relying on discrimination thresholds, and the use of statistical distributions to model intraspecific color variations. Results show that whilst an experienced insect observer can frequently make accurate discriminations between mimic and rewarding flowers, intraspecific signal variability leads to overlap in the perceived color, which will frequently confuse an inexperienced pollinator. This new perspective provides an improved way to incorporate pollinator decision making into the complex field of plant-pollinator interactions.

Keywords: mimcry, color modeling, orchid, signal detection, pollination, honeybee

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1. INTRODUCTION

Those walking through a forest in early spring cannot help to notice the burst of biological activity evidenced by the wide array of different sounds, aromas and movement. However, due to the particularities and complex architecture of our own senses, the vast palette of color produced both by animals and plants will quickly catch our eyes and very likely drive our attention. Human fascination with colors presented by nature is old with formal writings on the topic dating back to Aristotle, one of the earliest naturalists, who pointed out that "*Whatever is visible is color*

and color is what lies upon what is in its own nature visible" (Aristotle, 1970). Whilst modern color science separates the philosophical aspects of color theory from its physical nature, there still remains several ways to interpret color: either as a purely physical property of objects, as a subjective experience of the observer; or, by acknowledging that the physical aspects of color drive the subjective experience of the observer (Hatfield, 2003). Whilst the study of color in ecological and evolutionary contexts are currently mainly driven by purely physical aspects, i.e., treating color as a trait, it is potentially important to also consider the perceptual aspects of color vision to better understand how animals use this information to drive their behavior (Bruce et al., 2003).

The evolution of flower coloration has become a recurring research topic in biology, and color is often used as measurable trait for understanding different plant-pollinator interactions. We now recognize that many animals see the world differently to us, as suggested by data on the spectral sensitivity of their photoreceptors (Kemp et al., 2015). For example, many hymenopteran pollinators are characterized by possessing a trichromatic visual system perceiving spectral radiation between about 300 and 650 nm (Peitsch et al., 1992), allowing them to perceive UV radiation invisible to us, but limiting their ability to discriminate long wavelengths, which we and at least some other primates can easily recognize as being "red." On the other hand, most birds have a tetrachromatic visual system that is often sensitive to ultraviolet and long wavelength radiation from about 350 to 700 nm (Hart and Hunt, 2007), allowing them to perceive spectral and non-spectral color stimuli (Stoddard et al., 2020). These differences suggest that different animals, including humans, very likely perceive the same object differently and as such all interpretations of a color signals should be made considering the specific characteristics of the visual system of the receiving animal (Cuthill et al., 2017).

In its most basic definition, the term trait is used to describe a measurable feature at the individual level, and as floral color can have a large impact on the fitness of a plant, it is considered a key functional trait (Violle et al., 2007; Phillips et al., 2020). When applied to plants, such as comparative studies testing for an association between colors and pollinator groups, some authors refer to color through visual attributes defined by human perception, such as brightness, saturation, and hue (Smith, 2014; Reverté et al., 2016). Whilst the use of these attributes has provided interestingly insights into the distribution of plant colors along spatial gradients (Gray et al., 2018), and its association with biotic and abiotic factors (Dalrymple et al., 2015, 2020; Reverté et al., 2016), it is still unclear if color attributes applicable to human vision are universal among animals and relevant for all species. Brightness, for example, is a confound to color perception (Kelber et al., 2003) and in primates during the early stages of visual processing the chromatic and achromatic information are separated (Livingstone and Hubel, 1988; Nassi and Callaway, 2009) into the magnocellular and pavocellular pathways. It is only latter that these pathways are integrated using multiple stages in the primate brain to enable the dynamic color perception including brightness that humans have (Nassi and Callaway, 2009). Currently there is no definitive proof that any non-primate animal processes brightness as a dimension of color vision, and thus using human perception to define traits of flowers that are not pollinated by primates is highly questionable. Therefore, there is a need to understand how pollinators perceive color signals.

With the exception of a few recent studies in plant-insect interactions (Shrestha et al., 2019), the perceptual aspect of vision, the brain's interpretation of a physical color signal (Cornsweet, 1971), is rarely considered in animal color studies (Endler and Mappes, 2017). Reasons for this are the scarcity of data on the complex relationship between the neurophysiological processing of color signals and behavioral responses triggered by these. With the exception of humans, the European honeybee (*Apis mellifera*) is the only animal model for which there is currently sufficient data allowing us to model the complex and dynamic perceptual aspect of color vision in real-world scenarios (Dyer, 2012).

Bees and other pollinating insects live and navigate in complex and constantly changing environments, where they have to continuously process visual information from target and distractors to make decisions, often several times per second (Spaethe et al., 2001). To better understand how a bee may perceive color information in different ways depending upon the context in which colors are encountered, it is important to (i) understand how color stimuli are sensed and stored in memory by a visual system, and (ii) that evidence shows that the reliability with which color information can be recalled from memory is dependent upon individual experience (Dyer, 2012). Thus, a bee cannot be regarded as an ideal observer with perfect acuity, memory and color discrimination capabilities. Instead, under a Darwinian framework, bees should be regarded as an animal acting for its own survival based on the sensory processing capabilities it has evolved.

Considering the effect of memory on color perception, signals from a stimulus are initially processed at a photoreceptor level by integrating spectral reflectance, illumination and relative photoreceptor sensitivities (Chittka, 1992; Vorobyev and Osorio, 1998; Spaethe et al., 2001; Kemp et al., 2015). When two differently colored stimuli are viewed side by side at exactly the same time a very precise color judgement can be made. This is termed simultaneous color discrimination and is analogous to when we want an exact paint or fabric match to a known model color, so we take a sample to view side by side with any potential candidate color. However, if we are required to make an evaluation of a model color to a sample that is spatially separated then the information captured by photoreceptors must be coded to memory and then when a subsequent comparison is made the color must be retrieved from memory to enable a judgement of whether the colors are indeed the same. This is termed successive color discrimination, and in both humans (Newhall et al., 1957) and honeybees (Dyer and Neumeyer, 2005) color judgements with successive viewing conditions are significantly poorer than when made simultaneously.

Regarding the effect of individual experience on color perception, psychophysics experiments on honeybees (Giurfa, 2004; Reser et al., 2012), bumblebees (Dyer and Chittka, 2004), and hawkmoths (Kelber, 2010) shows that the accuracy with which an individual insect can make such color judgements is

dependent upon the level of experience with respective stimuli. Specifically, if an insect has only experienced one type of rewarding model color, which is termed *absolute conditioning*, color discrimination is subsequently relatively coarse when presented with similar alternative colors in a test. However, if an individual insect has the opportunity to learn a rewarding model color relative to a similar distractor, then learning occurs which results in changes in the brain and the enablement of long term memory (Dyer and Garcia, 2014; Sommerlandt et al., 2016).

Effects of memory and experience on color perception can be quantified by means of a function predicting the probability of accurate discrimination based on color similarity between two stimuli (von Helversen, 1972). Such a function has been formally derived from behavioral data (Dyer and Neumeyer, 2005) for honeybees and bumblebees considering simultaneous viewing condition (Garcia et al., 2017) and absolute conditioning (Garcia et al., 2018), but can also be formulated for successive color discrimination from behavioral data (Dyer and Neumeyer, 2005).

If color rather than spectral reflectance is a functional trait, the predicted accuracy of discrimination between two stimuli should be unaffected by context and/or memory as the observer judgement of the difference should be based solely on the stimulus' physical properties. Rejection of this null hypothesis would suggest that perception prevents the generalization of conclusions based on purely physical aspects of color signals. A biological system for testing this hypothesis is that of deceptive orchids where a non-rewarding species closely resemble a rewarding flower (Peter and Johnson, 2008; Jersáková et al., 2016). In this scenario, resemblance between mimic and model should be close enough that pollinators are sometimes unable to reliably discriminate between them (Jersáková et al., 2016). Specifically, here we use published data from Peter and Johnson (2008) on petal color from the mimic *Eulophia zeyheriana* (Orchidaceae) and the rewarding flower *Wahlenbergia cuspidata* (Campanulaceae) (**Figure 1**) to test this hypothesis. In the absence of color discrimination data for the *Lipotriches* bee pollinating these species, we used color discrimination data from *Apis mellifera*, a model hymenopteran pollinator, as it is known that trichromatic color vision is phylogenetically conserved in bees (Briscoe and Chittka, 2001). Whilst the precise effect of color similarity on discrimination accuracy may differ between species, data from Australian and Neotropical singless bees (Garcia et al., 2017), and more recently pollinator flies (Hannah et al., 2019), suggest that color discrimination by insects can be accurately described by continuous functions of different shape. So whilst the precise color discrimination capabilities of *Lipotriches* may differ from those observed in *Apis*, data from the latter species serves as a valid example of the general model describing the effects of cognition and viewing condition on color discrimination as theoretically predicted by von Helversen (1972).

Accuracy to discriminate between two stimuli based on their color similarity can be used as an indirect measurement of task's difficulty. For example, an accuracy of 0.75 means that there is a chance of 0.25 for a bee to make an error. In other words, a bee will fail to discriminate a stimulus from a distractor about once in every four choices. Under our null hypothesis, the probability of discrimination between the mimic and model flower colors should remain at the same level independent of experience and viewing conditions: i.e., the probability of discriminating two stimuli based on their color difference should be independent of conditioning and viewing conditions. As an alternative hypothesis we propose that an acquired tolerance to "perceptual noise" arising from color variability on petals of the rewarding species (Garcia et al., 2018) affects the probability of a pollinator accurately discriminating between rewarding and mimic flowers. When a bee searches for a target it should be able to detect and discriminate it among a set of options, potentially including non-rewarding distractors. However, the color signal produced by rewarding "target" flowers is also variable and likely discriminable by bees (Paine et al., 2019). Therefore, a pollinator should balance the probability of rejecting a correct flower as a result of only accepting a narrow range of color variants of their target, i.e., increase their possibility of a Type I error. To decrease the chances of committing Type I errors, a bee could increase its tolerance to accept a wider range of the target's color variants. This solution, however, would then increase its chances of accepting non-rewarding mimics resulting in an increase of its Type II error (Endler and Mappes, 2017).



FIGURE 1 | Flowers used in our study to understand pollinator decision making depending upon experience and viewing conditions. **(A)** Rewarding *Wahlenbergia cuspidata* (Campanulaceae) with mean flower size of 13.5 mm (Brehmer, 1915), and **(B)** rewardless *Eulophia zeyheriana* (Orchidaceae) with mean flower size of 8.4 mm (Rolfe, 1913). Photos by C. Peter.

Lichtenberg et al. (2020) presented a theoretical model explaining the complexity of this scenario using univariate probability density functions (PDFs) to describe the effects of signal variability in both mimic and rewarding species. This is an interesting approach as PDFs better describe the distribution of flower colors in the wild in similar way to that encountered by a foraging bee. The area where two PDFs overlap creates a "confusion" region: signals falling within this area will be ambiguous and potentially difficult to discriminate by a pollinator as it could correspond to either the mimic or an infrequent color signal variant of the rewarding species

(Lichtenberg et al., 2020). Here we apply and extend this theoretical model to color signals of wild plants to test if tolerance to perceptual noise, resulting from an increase in discrimination ability through experience and bee pollinator viewing conditions, is a likely explanation to the success of mimic orchid species.

2. MATERIALS AND METHODS

2.1. Case Model: Flower Mimicry in Floral Deception

To test our null hypotheses of whether color signals are a constant trait rather than variable perception if values change depending upon bee viewing conditions, we used reflectance data from *Wahlenbergia cuspidata* and *Eulophia zeyheriana* published by Peter and Johnson (2008) (**Figure 1**). *E. zeyheriana* is a terrestrial orchid that is restricted to grasslands of the Drakensberg Mountains of the summer rainfall region of South Africa (Johnson and Bytebier, 2015). While the flowers are self-compatible, a pollen vector is required for effective pollination to occur. *E. zeyheriana* (Orchidaceae) is pollinated by males of a single undescribed species of *Lipotriches* bee (Halictidae). For a human observer, the rewardless flowers closely resemble those of the co-flowering nectar producing species *W. cuspidata* (Campanulaceae) (**Figure 1**). At these sites *W. cuspidata* is a major food source of the *Lipotriches* bees. The bees are thought to be attracted to *E. zeyheriana* through their similarity in color and overall floral shape to *W. cuspidata*. Both species have prominent petals that appear blue-violet to the human eye. The pollen presenter in *W. cuspidata* is white to the human eye and is similar in color to the white papilose area of the labellum of *E. zeyheriana*. While male bees do not collect pollen, the white area of the labellum of *E. zeyheriana* may be important for mimicking the overall floral pattern of *W. cuspidata*. Measurements of spectral reflectance revealed that the petals of both species are similar and located in the blue-UV segment of the hexagon model of bee vision (Peter and Johnson, 2008). An experiment where flowers were painted with a UV absorbing mixture revealed that flowers became less attractive to pollinators when they did not reflect UV, suggesting that floral color plays an important role in pollinator attraction. Scent is unlikely to be used as a luring cue as bees show no response to scent extracts of *Wahlenburgia* flowers (Welsford and Johnson, 2012). Further, reproductive success of the orchid was greater in close proximity to the model species (Peter and Johnson, 2008).

2.2. Absolute and Successive Viewing Color Discrimination Functions

We modeled color discrimination functions for *Apis mellifera* from isoluminant "blue" and "yellow" stimuli considering successive viewing and absolute conditioning using data from behavioral experiments (Dyer and Neumeyer, 2005; Garcia et al., 2018). Both functions describe the probability of accurate discrimination for increasing color differences, here expressed as distance in the hexagon space (Chittka, 1992), by means of a non-linear expression. Functions were fitted using a least-squares regression using the methods by Garcia et al. (2017) to model the color discrimination function for this species when stimuli were observed simultaneously. A separate function was modeled when color discrimination occurs under absolute conditioning; i.e., when bees learn the target stimulus in the absence of a distractor.

We fitted a non-linear mixed effect model using the package nlme for the R environment for statistical computing to produce the successive discrimination functions for the "blue" and "yellow" color stimuli. As the response variable we used the proportion of correct choices made by $n = 5$ and $n = 4$ bees when discriminating a reference stimulus from a set of nine different blue and yellow distractors, respectively varying in color similarity to the reference. See Dyer and Neumeyer (2005) for a complete description of the behavioral experiment and stimuli. Color dissimilarity between each reference/distractor pair, expressed as Euclidean distance in the hexagon color space (Chittka, 1992), was used as an independent variable in the model. Bee ID number was included as a random term in each model to account for the multiple measurements collected from each individual bee.

For the absolute discrimination behavioral experiment, a total of six different color stimuli were tested, comprised of three samples from the "yellow" and "blue" stimulus sets. Experimental data showed the same behavioral response from bees to larger color differences, so only three stimuli pairs were tested for each color. To ensure a robust fit, responses from the $n = 9$ tested bees to the six stimuli were pooled and used as response variable, so no random term was included in this model.

If color distance represents a measurable trait, the same mathematical function can be used to describe the relationship between color difference (ΔC) and probability of accurate discrimination (π) under absolute conditioning and successive viewing conditions. We formally tested this hypothesis by initially fitting a three (Equation 1) and four (Equation 2) parameter logistic functions to each data set, and subsequently used a likelihood ratio test (LRT) to compare between the two models. If the LRT test was not significant for an $\alpha = 0.05$, we selected the simpler function following standard model selection procedures (Faraway, 2006). Under the null hypothesis, we expected that the two datasets can be modeled by the same type of function.

$$\pi = \frac{M_o K}{Mo + (K - Mo)\exp(-r \cdot \Delta C)} \quad (1)$$

$$\pi = \frac{Mo + (K - Mo)}{1 + \exp(\frac{xmid - \Delta C}{r})} \quad (2)$$

In the three parameter function (Equation 1), K defines the upper limit of the function, M_o represents the ΔC value at which the function begins to increase rapidly, and r gives the increment rate. In the four parameter model, K and M_o indicate values of the upper and lower asymptotes of the function, respectively; r describes magnitude of the increment rate, and *xmid* determines the value of ΔC corresponding to the first inflection point of the curve (Garcia et al., 2017).

Even if the functions modeling color discrimination under absolute conditioning and successive viewing have the same

number of parameters, it is possible that the coefficients shaping each function differ thus suggesting a different relationship in each case. Mechanistically, such changes would reflect how the brain of honeybees changes and develops long term memory depending upon conditioning (Sommerlandt et al., 2016). Under the null hypothesis, probability of accurate discrimination predicted by both functions should not be significantly different in spite of shape differences; therefore, we also tested for equality between the mean probability of accurate discrimination predicted by the absolute and successive functions using a bootstrap test for equality of means (Hall and Hart, 1990; Efron and Tibshirani, 1993). For completeness, we also compared the shape of absolute conditioning and successive viewing conditions against the function for simultaneous discrimination published by Garcia et al. (2017). All bootstrap tests were performed with 100,000 samplings with replacement.

2.3. Spectral Measurements

For full methodology see Peter and Johnson (2008). However, briefly, the reflectance spectra of floral parts of the two species were measured using an Ocean Optics S2000 spectrophotometer (Ocean Optics, Dunedin, FL, USA), coupled to an Ocean Optics Mini-D2T light source. We measured spectra of $n = 25$ adaxial petal surfaces of separate flowers for both *E. zeyheriana* and $n = 26$ *W. cuspidata* flowers, as well as $n = 22$ point samples of the prominent white papillose area of the labellum of *E. zeyheriana* and $n = 22$ samples of the pollen-covered pollen presenter of male-phase *W. cuspidata* flowers. Raw spectral data were binned into 10 nm intervals between 300 and 650 nm using the piece wise cubic Hermite interpolating Polynomial routine for Python 3.7. Spectra was subsequently modeled in the hexagon color space by Chittka (1992) assuming an average green leaf as adaptation background (Bukovac et al., 2017) and an illumination typical of a clear midday open sky in the Northern hemisphere (Judd et al., 1964) expressed as photon flux using custom code written for Matlab release 2017 (The Mathworks, USA).

2.4. Color and Statistical Modeling

The particular conditioning of a pollinating bee and target viewing conditions, either simultaneous or successively, determines the minimum color difference it requires to accurately discriminate between two samples (Dyer and Chittka, 2004; Giurfa, 2004). However, color variability in the observed flowers determines the frequency by which the pollinator will encounter a flower pair whose color difference is low enough that they cannot be reliably discriminated between.

Color signals produced by flowers and perceived by trichromatic pollinators, such as most bees (Briscoe and Chittka, 2001), are modeled as bivariate variables. More specifically, the spectral profile making up the color signal can be modeled in a two dimensional space (Shrödinger, 1970), such as the Maxwell triangle (Neumeyer, 1980), hexagon color space (Chittka, 1992), or other alternative models each with their own set of assumptions (see Renoult et al., 2017 for a review). For example, modeling the different spectral measurements collected from pollen of *W. cuspidata* and labella of *E. zeyheriana* produces two clouds of points whose shape, distribution and sparseness correspond to differences in their spectral profiles (**Figures 2A,B**).

The colors more frequently observed on each species will be clustered together in the same region of color space. If variability is low, most samples will be located in a small area of color space resulting in PDF high density (d) values. On the contrary, if variability is high, colors will be distributed in a wider area of color space resulting in a lower density. In a two dimensional space PDFs are not represented as curves, but as mound-shaped surfaces where the breadth and height of the peak is given by density of the most frequently observed loci (**Figures 2C,D**).

The most commonly used multivariate distribution to model two dimensional data is the (bivariate) joint normal distribution where the two variables are described by the same univariate distribution. This condition, however, is rarely observed in natural samples as flower colors in plant populations tend to be clustered in particular areas of color space (Chittka and Menzel, 1992; Dyer et al., 2012; Shrestha et al., 2014). A solution to model complex PDFs is modeling the joint cumulative distribution function for two continuous variables (marginals), each described by a different distribution, and their dependence structure independently through a copula (Genest and Favre, 2007).

Marginals and copulas describing the PDFs for petals, pollen presenter and labella of *W. cuspidata* and *E. zeyheriana* were fitted by maximum likelihood employing the package Vinecopula (Nagler et al., 2019) for the R language and environment for Statistical Computing (R Core Team, 2020). Marginals were fitted by maximum likelihood using the package fitdistrplus (Delignette-Muller and Dutang, 2015) for R, and tested for goodness of fit using the Anderson-Darling test available in the ADGofTest package (Bellosta, 2011) for R.

2.5. Likelihood of Discrimination in the Presence of Color Noise

In a symmetrical PDF, such as a bivariate normal distribution, frequency of observed loci could be predicted from the analytical expression describing this distribution as done in most parametric multivariate analysis techniques (Johnson and Wichern, 2007). However, such an approach cannot always be implemented when PDFs are modeled by copulas. To identify typical, less frequent and rare colors from the different plant species, we generated 100,000 random samples from their corresponding PDFs and calculated their density using functions available in the package copula (Kojadinovic and Yan, 2010) version 1.0-0 for R. For each species we subsequently obtained density values corresponding to the 0.9, 0.55, 0.45, 0.25, and 0.15 probability quantiles. As larger density values correspond to a higher probability, we assigned as typical colors loci whose density values were higher than the 0.9 quantile; less frequent colors as those whose probability of occurrence is between 0.45 and 0.55; and as rare, colors with density values corresponding to a probability between 0.15 and 0.25.

If probability density functions can tell us the likelihood of observing a flower of any given color for a species, discrimination functions will predict the probability with which given flower

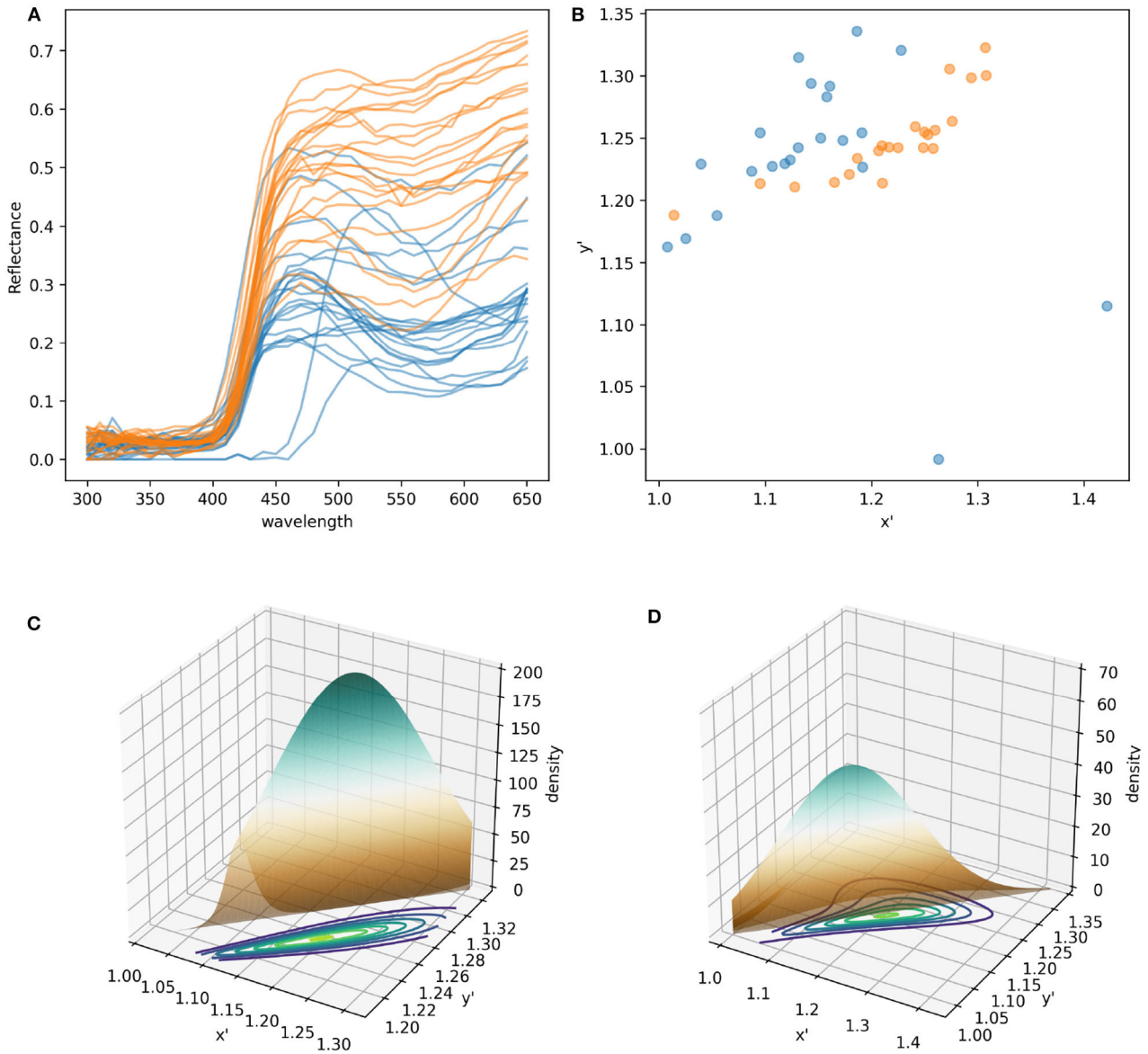


FIGURE 2 | Modeling and statistical analysis of color variability in flower samples. **(A)** Reflectance profiles of pollen grains from *W. cuspidata* (blue) and labella region of *E. zeyheriana* (orange). **(B)** The same samples following modeling in the hexagon color space and translating the original x-y coordinates to an alternative x′ y′ coordinate system to ensure that all values are positive. Surfaces describing the probability density functions corresponding to the orange **(C)** and blue **(D)** cloud of points. Note how differences in variability are reflected in different maximum density values in the two PDFs as indicated by the surface's maximum height. Contour maps of **(C,D)** are two-dimensional representations of their respective surfaces. On these maps each contour represents different density values in analogous way to physical maps that use contours to represent altitude variations in the landscape.

colors can be discriminated given a bee's experience and viewing conditions. We can use both of these functions to predict if pollinating bees can perceive as being different typical, less frequent and rare flower colors. To answer this question, we applied the same sampling method for each species PDF and classified them as typical, less frequent or rare based on their density values. Then, we calculated the Euclidean distance between loci pairs belonging to each group and obtained their mean distance, and used functions to obtain their predicted probability of discrimination.

We used a similar approach to predict the probability with which a bee can discriminate between likely colors of two different species. We generated random loci from the respective PDFs, and calculated the Euclidean distance between each loci pair. We then used the color discrimination functions to obtain the probability of accurately discerning between each

stimulus pair considering absolute conditioning, successive and simultaneous viewing conditions.

3. RESULTS

3.1. Color Discrimination Function Under Successive Viewing Conditions

We initially tested if a function with four parameters was necessary to obtain a better fit of data describing discrimination under successive viewing conditions, or if a simpler alternative with one less coefficient could provide the same fit using a likelihood ratio (LRT) test. We found no significant difference in the goodness of fit provided by a three or four parameter logistic function for either the blue or yellow stimuli ($\chi^2_{blue} = 0.161$, $P = 0.688$; $\chi^2_{yellow} = 1.73$, $P = 0.188$), so the former function was used to model the two data sets as it is mathematically simpler.

TABLE 1 | Coefficients (*K*, *r*, *Mo*) defining the shape of a three parameter logistic curve (Equation 2) describing the probability of accurate color discrimination by *Apis mellifera* when successively viewing "blue" and "yellow" stimuli increasing in color dissimilarity.

	K	*r*	*Mo*
Blue	0.990 (0.969, 1.01)	53.8 (45.3, 66.3)	0.528 (0.474, 0.571)
Yellow	0.951 (0.913, 0.989)	47.2 (35.7, 69.5)	0.555 (0.420, 0.651)

Values in parentheses are the 95% confidence intervals for each coefficient.

For the "blue" and "yellow" color stimuli we initially fitted a non-linear model including a random term for each one of the three parameters and alternative reduced models including only fixed terms for each term, followed by LRT comparisons. This method allowed us to identify which of the three parameters was significantly varying across individuals so that it should be included as a random term in the final model (Pinheiro and Bates, 2000).

For the "blue" and "yellow" stimuli functions the second term showed the highest variability across individuals so it was included as a random term in both models. Values for coefficients (*K*, *r*, *Mo*), defining the shape of each discrimination function are provided in **Table 1**, and a graphical representation of the two functions is given in **Figure 3**.

For the absolute color discrimination function, a four parameter logistic model provided a significantly better fit than the simpler, three parameter alternative ($\chi^2 = 4.69$, $P = 0.030$) so the former was used to fit the data. Coefficients describing the absolute color discrimination function for *A. mellifera* are provided in **Table 2**, and a graphical representation of the function is given in **Figure 4**.

Mean probability of accurate discrimination predicted by the absolute conditioning and successive viewing conditions were significantly different from each other ($t_{Ho} = -22.0$, $P < 0.001$). Likewise, significant differences in predicted accuracy were observed between the absolute and successive functions ($t_{Ho} = -23.5$, $P < 0.001$), and between successive and simultaneous conditions ($t_{Ho} = -16.5, P < 0.001$). This result evidences a significant effect of experience and memory on the perception of color differences by *A. mellifera*.

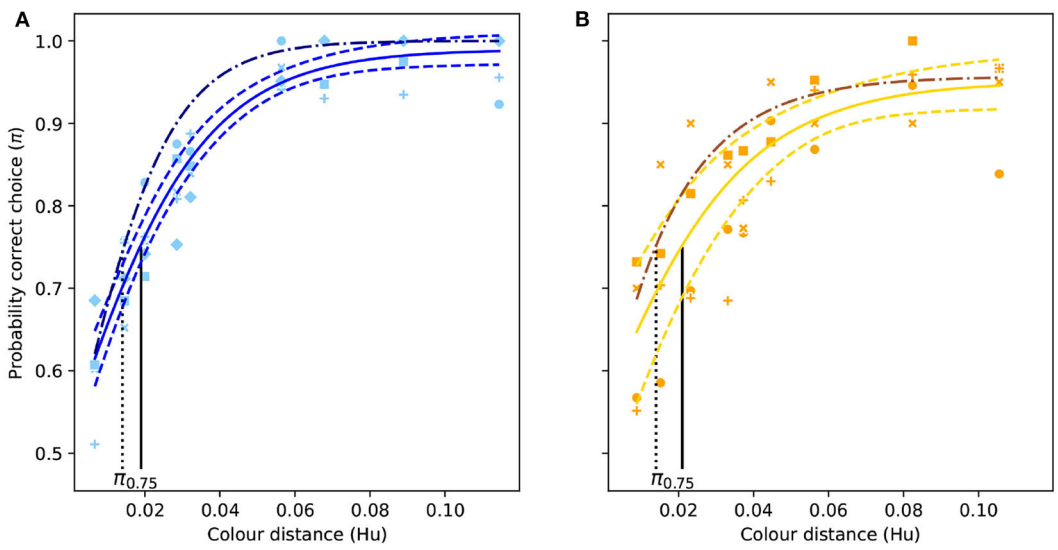


FIGURE 3 | Color discrimination functions (solid colored lines) for deferentially-conditioned *Apis mellifera* when successively viewing different isoluminant "blue" **(A)** and "yellow" stimuli **(B)** varying in color similarity here expressed as Euclidean distance in the hexagon model (Hu) for hymenopteran vision (Chittka, 1992). Vertical black line indicates the color distance at which a bee observer will discriminate between a pair of color stimuli with a probability of 0.75 (π_{75}). Dashed lines represent the 95% confidence intervals for their respective function, and the dash dotted line represent the color discrimination function for simultaneous viewing for the same stimuli (Garcia et al., 2017). Markers represent the proportion of correct choices made by individual bees.

TABLE 2 | Coefficients (*Mo*, *K*, *xmid*, and *scal*) defining the shape of a four paramter logistic curve (Equation 1) describing the probability of accurate color discrimination by *Apis mellifera* when discriminating "yellow" and "blue" stimuli following absolute conditioning.

	K	*Mo*	*xmid*	*scal*
Absolute	0.842 (0.766, 0.919)	0.441 (0.377, 0.505)	0.081 (0.076 0.086)	0.009 (0.004, 0.015)

Values in parentheses are the 95% confidence intervals for each coefficient.

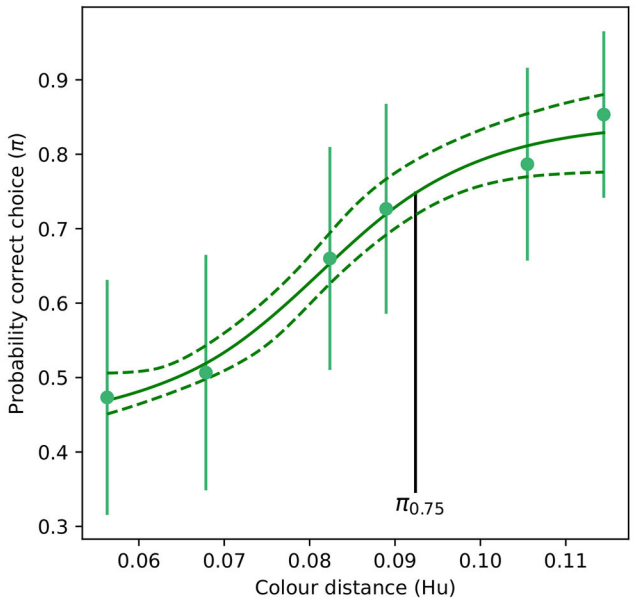


FIGURE 4 | Color discrimination function for inexperienced *A. mellifera* under absolute conditioning. The vertical black line indicates the color distance at which a bee observer will discriminate between a pair of color stimuli with a probability of 0.75 (π_{75}). Note that for an inexperienced bee, larger color distances are required to achieve the same probability of accurate discrimination than for the experienced forager modeled in **Figure 3**. Dashed lines indicate the 95% confidence intervals for their respective function. Markers represent mean responses from $n = 9$ bees. Whiskers indicate the standard error of the mean proportion of correct choices.

3.2. Effect of Color Difference and Variability

The color of lateral petals of the orchid *Eulophia zeyheriana* and the sympatric rewarding species *Wahlenbergia cuspidata* appear blue to the human eye and fall between the blue and blue-UV sectors of the color hexagon (**Figure 5**). Consequently the "blue" discrimination function was used to model this system.

Mean color differences between all petals samples of *E. zeyheriana* and *W. cuspidata* was 0.056 (Hu) ± 0.032 (standard deviation). Such a color difference cannot be discriminated by an inexperienced bee as the probability of accurately discriminating between such a stimuli is of 0.5. However, an experienced bee can discriminate between petals of the two species with an accuracy of 0.949 and 0.987 when comparing them in succession or simultaneously, respectively. Color differences between the labellum of *E. zeyheriana* and the pollen presenter of *W. cuspidata* are easier to differentiate by a bee. Mean color differences between these two stimuli is equal to 0.138 ± 0.074 (Hu), which can be discriminated by an inexperienced bee with an accuracy of 0.841. This value increases to 0.99 for an experienced bee comparing between these stimuli either successively or simultaneously. Altogether, the results indicate that the perception of a given color difference changes with experience of the pollinator and is also context dependent, hence perception prevents interpreting the color signal as a trait. Our results suggest that spectral reflectance data should always be interpreted in a specific context, thus rejecting the null hypothesis that color interpretation is independent from viewing conditions and experience; we thus proceed to test the color noise alternative.

To model signal noise produced by color variability, bi-variate probability density functions (PDFs) were fitted to data from petals, pollen and labellum from rewarding and mimic, respectively, using copulas (**Figure 6**). Parameters defining each PDF are given in **Table 3**.

Low color noise on petals of both species make typical, less frequent and rare colors more similar to each other and thus harder to discriminate by an insect pollinator. Indeed, mean color difference between typical and rare petal colors of *E. zeyheriana* and *W. cuspidata* is of about 0.025 and 0.051 Hu, respectively, which are unlikely to be discriminated by an inexperienced bee forager, here modeled using the discrimination function for absolute conditioning. However, an experienced pollinator would be able to discriminate between typical, less frequent and rare petal colors of *E. zeyheriana* with an accuracy between 80 and 90% when viewing them successively. This value increases to about 90% if colors are viewed simultaneously (**Table 4**). Larger color difference in petals of *W. cuspidata* makes discrimination between typical and less frequent colors more likely with a probability of an accurate discrimination higher than 0.9 for successive and simultaneous viewing conditions.

Labellum and pollen color loci show higher variability than petals in both *E. zeyheriana* and *W. cuspidata*. Mean color difference between typical and rare colors of *E. zeyheriana* labella is ∼0.06 Hu, whilst differences in pollen color of *W. cuspidata* is ∼0.086 Hu. Whilst differences between labella cannot be discriminated with an accuracy higher than 50% by inexperienced foragers, our model predicts that the same observers would be able to discriminate between typical and less frequent pollen colors about 70% of the time. Experienced foragers are predicted to discriminate between typical and less frequent colors of labella and pollen with an accuracy higher than 90% when viewing these stimuli either successively or simultaneously (**Table 4**).

The confusion region produced by overlap of probability density functions (PDFs) from petals of *E. zeyheriana* and *W. cuspidata* suggests that the most frequent colors produced by each species roughly occupy the same area of color space (**Figure 6**). In a scenario where both mimic and rewarding species have a similar abundance, bees searching for the rewarding species will very likely find flowers of the mimic whose color is very similar to the most frequently displayed by

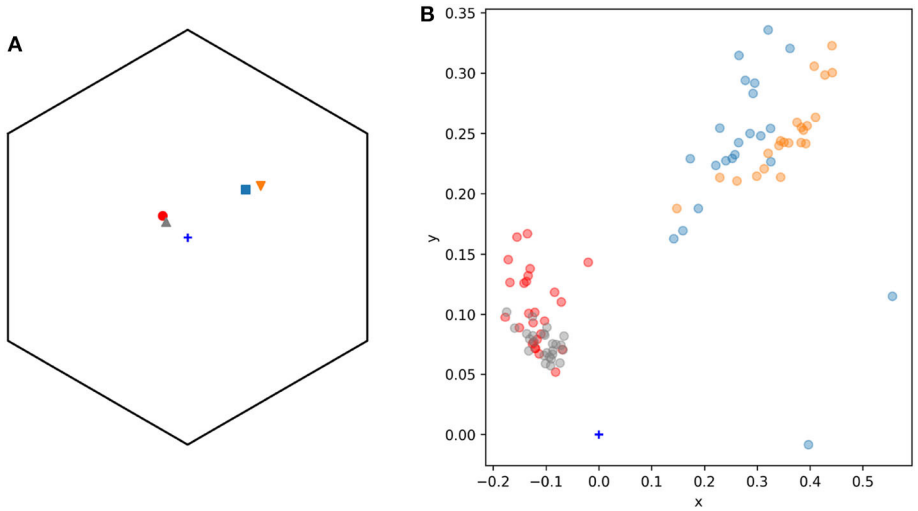


FIGURE 5 | General view **(A)** and detail **(B)** of the $n = 95$ spectral samples used to model adaxial lateral petals ($n = 25$, triangular markers) and labella ($n = 22$, inverted triangles) of the mimic orchid *Eulophia zeyheriana*, and adaxial petals ($n = 26$, circle markers) and pollen ($n = 22$, square markers) of the rewarding species *Wahlenbergia cuspidata*. **(B)** Shows a detail of **(A)** highlighting color variability in sampled regions. Marker colors were selected to ease visualization: gray (lateral petals *E. zeyheriana*), red (petals *W. cuspidata*), orange (labella *E. zeyheriana*), and blue (pollen *W. cuspidata*). Color space's center is indicated by a blue cross marker.

rewarding flowers. Indeed, the median color distance observed between petals of the two species is of about 0.053 Hu, which cannot be discriminated by an inexperienced bee. However, both colors can be identified as being different with a probability of 0.94 by an experienced bee if both targets as seen successively, and with a probability of 0.98 if seen simultaneously. Furthermore, 87.6% of comparisons between the lateral petals of the orchid flowers and petals of the rewarding species are below the 75% discrimination threshold for an inexperienced bee, although this proportion falls to 7.2% when considering an experienced forager observing petals from the two species and to 4.3% when flowers are observed simultaneously. The range of color distances for petals of *E. zeyheriana* and *W. cuspidata*, along with their associated probability of accurate discrimination by either inexperienced or experienced forager bees, is presented in **Table 5**.

Labella and pollen presenter color in *E. zeyheriana* and *W. cuspidata*, respectively, show a higher variability than petal color as indicted by the maximum density values of their PDF (**Figure 6**). Such a variability results in larger median color differences between these targets (0.110 Hu) than those observed for petals, in spite of presenting a larger confusion region in color space (**Figure 6**). This increase is the result of a higher chance of observing less frequent labella and pollen presenter colors, thereby facilitating discrimination for both inexperienced and experienced foragers (**Table 5**). Indeed, 41% of color comparisons between labellum and pollen colors will be below the 75% accuracy threshold for an inexperienced bee, whilst 3.1% of these comparisons will be below this level when considering an experienced bee observing both stimuli successively. The proportion of comparisons below the accuracy threshold diminishes to 1.8% when stimuli are observed simultaneously.

4. DISCUSSION

When using color distance, researchers seek to infer from this metric if a particular flower color signal has an effect on plant fitness. Whilst this metric can potentially describe differences in the physical nature of a color signal between flowers, it cannot predict unambiguously if such a difference is perceivable by a pollinator.

When answering questions about the behavioral response of an animal to perceived color differences, discrimination functions provide a more realistic prediction of what an animal may perceive and its response. Compared to morphological traits, such as shape, length, and width of advertising floral parts measured with precise instruments (Violle et al., 2007), the interpretation of color difference by an animal brain is frequently context dependent and thus not a trait. In the current manuscript, we show that considering absolute conditioning, an inexperienced bee would be able to discriminate a color difference of about 0.09 Hu with an accuracy of 75%. However, after a bee has acquired more experience it will be able to discriminate the same color difference with an accuracy of almost 100% (**Figures 3**, **4**). As such, we show that for a given color distance it is more appropriate to discuss the likelihood that an inexperieced or experienced pollinator is deceived by the mimic, rather than using a single color distance threshold.

Functions defined by Equations (1) and (2) and corresponding coefficients (**Tables 1**, **2**), allow for the construction of modeling tools describing color discrimination by honeybees considering multiple viewing conditions including absolute conditioning (**Figure 4**), and simultaneous or successive color discrimination (**Figure 3**). Significant difference in the predicted probability of accurate discrimination by respective functions for the same color distance rejects the null hypothesis of equality and

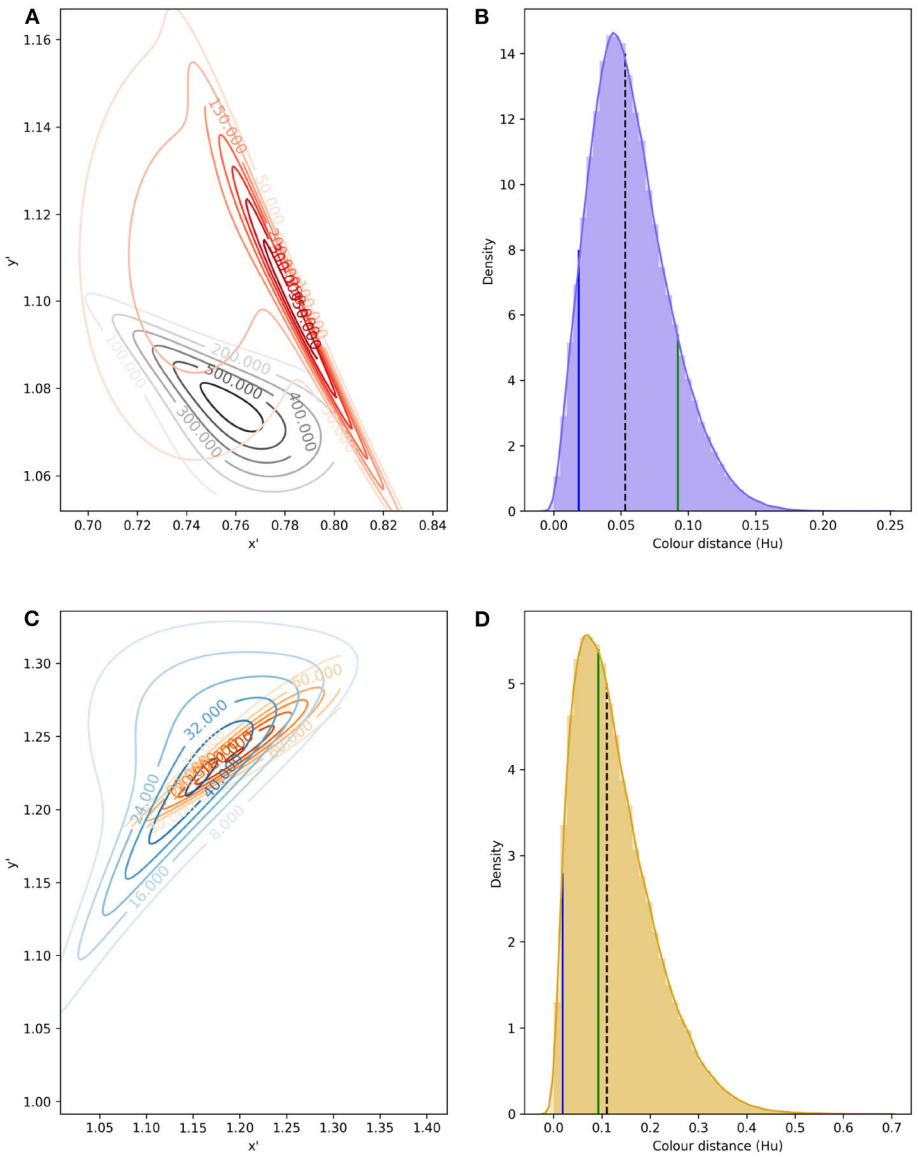


FIGURE 6 | Confusion region in hexagon color space produced by the overlap of bivariate PDF describing variability in color signal produced by lateral petals of the orchid *E. zeyheriana* and petals of the rewarding flower *W. cuspidata* **(A)**; in addition to labella and pollen presenter from respective species **(C)**. Numbers on the contour lines indicate the various density values for each PDF, larger magnitudes represent areas of color space where most samples occur for each species. All loci were translated from their original hexagon x-y coordinates into a new set of coordinates x′ and y′ where all values are positive. Distribution of Euclidean distances obtained after drafting 100,000 random samples from petal **(B)** and labella/pollen distribution **(D)**. In **(B,D)**, black, dashed lines represent the median distance; solid, green lines indicate the color difference which can discriminated by an inexperienced bee with an accuracy of 75%; and blue lines indicate color distance for the required by an experienced forager to attain the same accuracy when observing both targets successively.

evidences an effect of both experience and conditioning on perception of floral color difference by a pollinating bee. This result thus indicates that though a flower's reflectance spectrum can be considered as a functional trait in some circumstances (Dalrymple et al., 2020), its interpretation by the brain of a pollinator cannot. Therefore, the *color sensation* experienced by a bee is frequently context dependant, and as such, cannot be quantified and compared as other purely physical traits. Interestingly, the use of functions like those presented in **Figures 3**, **4** produce data from physical traits that are compatible with a signal detection theory (Endler and Mappes, 2017; Lichtenberg et al., 2020), which better predict bee behavioral responses when foraging in the presence of rewarding targets and non-rewarding distractor flowers.

Studies of plant-pollinator interaction can benefit from considering insect perception of flower color, as this can provide better explanations of the relationship between the spectral color signal as measured by a spectrometer and animal behavior.

TABLE 3 | Parameters for the different elements describing the probability density function (PDF) of color loci for the different flower parts considered.

Stimulus	Component	Distribution	Parameter (SE)
W. cuspidata petals	x′	Gamma	Shape = 491 (136)
			Rate = 659 (183)
	y′	Gamma	Shape = 1270 (353)
			Rate = 1150 (320)
	Copula	Tawn II 270°	Par 1=-9.13 (4.75)
			Par 2=0.244 (0.030)
W. cuspidata pollen presenter	x′	Gamma	Shape = 182 (54.9)
			Rate = 159 (48.0)
	y′	Weibull	Shape = 22.8 (3.86)
			Scale = 1.26 (0.012)
	Copula	Tawn II 180°	Par 1=3.61 (0.846)
			Par 2=0.490 (0.110)
E. zeyheriana lateral petal	x′	Normal	Mean = 0.761 (0.006)
			Std = 0.027 (0.004)
	y′	Normal	Mean = 1.08 (0.002)
			Std = 0.012 (0.002)
	Copula	Joe 90°	Par 1 = −2.15 (0.552)
E. zeyheriana labellum	x′	Normal	Mean = 1.22, (0.015)
			Std = 0.070 (0.011)
	y′	Gamma	Shape = 1370 (423)
			Rate = 1100 (339)
	Copula	Survival Gumbel	Par 1= 4.19 (0.764)

Each PDF is defined by the univariate distribution of the values for x and y color coordinates in the hexagon space (marginals) and their join distribution modeled by a specific copula type. SE denote the standard error for each parameter value.

TABLE 4 | Probability of a honeybee discriminating between pairs of typical (typ.), less frequent (lfq.) or rare (rar.) colors for various flower regions of *E. zeyheriana* and *W. cuspidata* under absolute conditioning, or when seeing targets successively or simultaneously.

Species	Region	Pair	*d*	Δ*C*	Abs	Succ.	Simul.
E. zeyheriana	Lateral petal	typ./lfq.	>590	0.025	0.500	0.806	0.873
		typ./rar.	$300 \leq d < 360$	0.035	0.500	0.874	0.938
		lfq./rar.	$100 \leq d < 166$	0.040	0.500	0.899	0.957
	Labellum	typ./lfq.	> 188	0.063	0.500	0.962	0.993
		typ./rar.	$87 \leq d < 109$	0.094	0.765	0.985	0.999
		lfq./rar.	$26 \leq d < 45$	0.100	0.759	0.986	1.00
W. cuspidata	Petal	typ./lfq.	> 250	0.051	0.500	0.937	0.982
		typ./rar.	$90 \leq d < 108$	0.070	0.532	0.970	0.996
		lfq./rar.	$32 \leq d < 53$	0.057	0.500	0.951	0.988
	Pollen	typ./lfq.	> 42	0.086	0.716	0.982	0.999
		typ./rar.	$19 \leq d > 23$	0.135	0.841	0.989	1.00
		lfq./rar.	$6 \leq d < 10$	0.146	0.842	0.990	1.00

Color dissimilarity is expressed as mean distance in the hexagon space (ΔC) in all cases. Typical, less frequent and rare colors where determined from density (d) values of the PDF corresponding to each sample.

For example, Peter and Johnson (2008) concluded that the orchid *E. zeyheriana* is a non-rewarding mimic of the rewarding flowers of *W. cuspidata*, based on several lines of evidence, including their color difference. When considering petal color variability from the two plant species we predict that about 25% of flower comparisons made by an inexperienced bees will be easy to discriminate (**Figure 6B**), whilst an experienced bee viewing the same colors successively would make perceptual errors <5% of the time (**Figure 6B**). Considering simultaneous viewing conditions, a bee would almost never make a perceptual error (**Table 5**), further reinforcing the contextual nature of color as a perceptual signal. Thus, the color modeling provides insights

TABLE 5 | Median and range of color distances (ΔC) observed after randomly drawing 100,000 samples from the PDF describing color variability of the petals and pollen of the rewarding plant *W. cuspidata*; and, lateral petals and labellum of its mimic orchid *E. zeyheriana*.

Flower region pair	ΔC	π_{abs}	π_{succ}	π_{simul}
Lateral petal/petal	0.053 (≈0, 0.244)	0.500 (0.500, 0.842)	0.941 (0.529, 0.989)	0.984 (0.500, 1.00)
Labellum/pollen	0.110 (≈0, 0.679)	0.826 (0.500, 0.842)	0.987 (0.535, 0.989)	1.00 (0.502, 1.00)

Third to fifth columns contain the predicted probability of discrimination for either absolute (π_{abs}) conditioning, successive (π_{succ}), or simultaneous (π_{simul}) conditions corresponding to the reported color distances based on the discrimination functions in ***Figure 3****.*

into how bee pollinators contribute to the pollination system in a dynamic way.

Classically, deceptive flowers are thought to rely on inexperienced insect visitors for pollination (Jersáková et al., 2006). Whilst our modeling confirms that inexperienced bees are often unable able to discriminate between mimic and model species (**Figure 4**), bees can readily distinguish these colors after acquiring some experience in spite of memory limitations (**Figure 3**). Honeybees under differential conditioning can learn to discriminate colors following 15 (Giurfa, 2004) to 50 choices for very similar stimuli (Reser et al., 2012), and these differences form memory lasting for at least 48 h days after initial testing (Dyer and Garcia, 2014). In the wild, however, such a learning does requiring visiting some mimics. Whilst the precise number of visits to either rewarding or mimic species by *Lipotriches* bees is unknown, it is very likely that hundreds of visits are done in the wild at least to rewarding flowers, suggesting that learning likely occurs in natural settings.

Considering that experienced bees are likely to discriminate between mimic and rewarding species (**Table 5**), an alternative explanation for the repeated visitation to flowers observed in the *E. zeyheriana*-*W.cuspidata* system (Peter and Johnson, 2008) is that bees develop a tolerance to color variability as a means to maintain flower constancy toward the rewarding species. Color variation in *W. cuspidata* is large enough that less frequent and rare colors are easy to discriminate from the more typical flowers (**Table 4**). Thus, bees visiting the rewarding species should have to develop a tolerance to "color noise" in order to identify the various colors displayed by flowers of this species (**Figure 5**). As color variability of the mimic is lower than the rewarding species, bees are likely to accept flowers of the mimic as potential variants of *W. cuspidata* (**Figure 6**). Such an outcome is consistent with signal theory predictions where an decrease in Type I errors results in an increase of the probability of making Type II errors (Endler and Mappes, 2017; Lichtenberg et al., 2020) highlighting the benefits of using PDFs to the study of plant mimicry.

Our empirical and statistical evidence partially addresses theoretical positions of pollinator generalization (Fields et al., 1991) and/or generalized food deception in orchids (Jersáková et al., 2006). Considering pollinator decision making, generalization refers to an animal responding to stimuli that differ in some dimension from a target stimulus (Fields et al., 1991; Aguiar et al., 2020). For example, inexperienced honeybees predominantly use simple elemental cues and will generalize to similar shapes (Horridge, 2009) or colors (Giurfa, 2004), whilst experienced bees show evidence of fundamentally different processing like statistical learning enables avoidance of perceptual errors resulting from generalization (Avarguès-Weber et al., 2020). Generalized food deception is a description of how some orchids show evidence of achieving pollination from insect species that may lack a capacity to overcome the limitations of reliable food identification via simple elemental cues (Steiner, 1998; Jersáková et al., 2006). Our understanding of the way these two types of generalization theories may interplay will likely benefit from the formal framework provided by the PDFs (**Figure 6**), and a better description on how pollinator experience mediates different choice criteria as modeled by the color discrimination functions (**Figures 3**, **4**).

Information from other floral traits, such as shape (Dyer and Chittka, 2004) and scent (Kunze and Gumbert, 2001; Leonard et al., 2011), have been shown to reduce information uncertainty in behavioral experiments and thus could help to set the balance between errors of Type I and II in the presence of color noise in deceptive orchid systems. Indeed, an important caveat in this field of research is that discrimination behavior is modeled based on color differences between matching floral parts. However, other cues are available to pollinators when making choices on visiting flowers. For example, there may be differences in color pattern that could inform foraging decisions, particularly if there is a variable model or multiple model species (e.g., Jersáková et al., 2016; Scaccabarozzi et al., 2018). Outside of color, floral odor (Leonard et al., 2011) and morphology (Howard et al., 2018) are used as cues to identify suitable food sources, and corolla shape has shown to be important for successful mimicry of flowers (Jersáková et al., 2012). More work is needed on how pollinators use colors together with other cues to discriminate between flowers and what this might mean for mimicry systems.

The approaches we present here allow for a more nuanced understanding of floral mimicry systems. Studies evaluating whether a rewardless plant uses a mimetic strategy often involve spectral reflectance measurements that are used to infer whether the mimic can be recognized by pollinators as different from the model based on a simple color discrimination threshold (Peter and Johnson, 2008). However, our use of functions (Equations 1 and 2) coupled with PDFs modeling illustrates that the ability of a pollinator to discern between model and mimic follows a non-linear relationship with color distance (**Figures 3**, **4**), and that the likelihood of successful discrimination is

greatly increased by experience foraging on the model/mimic. The use of discrimination functions combined with PDFs for modeling flower color variability represents a new solution for understanding other systems, such as: (i) mimicry of multiple models (Jersáková et al., 2016; Scaccabarozzi et al., 2018), (ii) systems where pollinators show some level of discrimination against the mimic despite effective pollination (e.g., de Jager et al., 2016), (iii) putative cases of generalized mimicry where color resemblance between mimic and model is very low (Jersáková et al., 2006), and (iv) the evolution of flower polymorphism (Kagawa and Takimoto, 2016). For example, our discrimination functions, and PDF of color variability can be used to model discrimination ability by pollinators to variable color based on real data in simulation experiments designed to explain the evolution of color polymorphism at species or population levels (Kagawa and Takimoto, 2016).

Further, this approach can be extended beyond mimicry systems, to understanding the foraging choices of pollinators when faced with a series of rewarding plant species, that may vary in the reward received compared with energy expended. A key challenge, however, for applying this approach will be the lack of knowledge of the psychophysics of pollinators outside of certain model species, although published data currently exists for three key bee pollinators (Garcia et al., 2017), and with these new methods it will be possible to push the frontiers of pollination biology and color signal evolution.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://doi.org/10.6084/m9.figshare.12899816.v1.

AUTHOR CONTRIBUTIONS

JG, AD, and RP developed the original conceptual framework of the study. CP collected the spectral data. JG analyzed and modeled the data. All authors contributed to writing and reviewing the manuscript.

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REFERENCES

Aguiar, J. M. R. B. V., Giurfa, M., and Sazima, M. (2020). A cognitive analysis of deceptive pollination: associative mechanisms underlying pollinators' choices in non-rewarding color polymorphic scenarios. *Sci. Rep*. 10:9476. doi: 10.1038/s41598-020-66356-4

Aristotle (1970). "On the soul," in *Sources Color Science, Chapter 2*, ed D. L. MacAdam (Cambridge, MA: MIT Press), 2–3.

Avarguès-Weber, A., Finke, V., Nagy, M., Szabó, T., D'Amaro, D., Dyer, A. G., et al. (2020). Different mechanisms underlie implicit visual statistical learning in honey bees and humans. *Proc. Natl. Acad. Sci. U.S.A.* 117, 25923–25934. doi: 10.1073/pnas.1919387117

Bellosta, C. J. G. (2011). *ADGofTest: Anderson-Darling GoF Test*. R package version 0.3.

Brehmer, W. (1915). Aufzählung der afrikan Arten von Wahlenbergia. *Bot. Jahrb. Syst*. 53, 126–127.

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol*. 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Bruce, V., Green, P., and Georgeson, M. A. (2003). *Visual Perception Physiology Psycology and Ecology, 4th Edn*. Hove: Psycology Press.

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017). Why background color matters to bees and flowers. *J. Comp. Physiol. A* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Chittka, L. (1992). The color hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of color opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colors and the insect pollinators' color vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/BF00188925

Cornsweet, T. (1971). *Visual Perception*. New York, NY: Academic Press.

Cuthill, I. C., Allen, W. L., Arbuckle, K., Caspers, B., Chaplin, G., Hauber, M. E., et al. (2017). The biology of color. *Science* 357:eaan0221. doi: 10.1126/science.aan0221

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2015). Birds, butterflies and flowers in the tropics are not more colorful than those at higher latitudes. *Glob. Ecol. Biogeogr*. 24, 1424–1432. doi: 10.1111/geb.12368

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower color are shaped by both biotic and abiotic factors. *New Phytol*. doi: 10.1111/nph.16737

de Jager, M., Newman, E., Theron, G., Botha, P., Barton, M., and Anderson, B. (2016). Pollinators can prefer rewarding models to mimics: consequences for the assumptions of Batesian floral mimicry. *Plant Syst. Evol*. 302, 409–418. doi: 10.1007/s00606-015-1276-0

Delignette-Muller, M. L., and Dutang, C. (2015). fitdistrplus: An R package for fitting distributions. *J. Stat. Softw*. 64, 1–34. doi: 10.18637/jss.v064.i04

Dyer, A. G. (2012). "Psychophysics of honey bee color processing in complex environments," in *Honeybee Neurobiology and Behavior: A Tribute to Randolf Menzel*, eds C. G. Galizia, D. Eisenhardt, and M. Giurfa (Dordrecht: Springer Netherlands), 303–314. doi: 10.1007/978-94-007-2099-2_23

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm color signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci*. 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., and Chittka, L. (2004). Fine color discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91, 224–227. doi: 10.1007/s00114-004-0508-x

Dyer, A. G., and Garcia, J. E. (2014). Color difference and memory recall in free-flying honeybees: forget the hard problem. *Insects* 5, 629–638. doi: 10.3390/insects5030629

Dyer, A. G., and Neumeyer, C. (2005). Simultaneous and successive color discrimination in the honeybee (*Apis mellifera*). *J. Comp. Physiol*. 191, 547–557. doi: 10.1007/s00359-005-0622-z

Efron, B., and Tibshirani, R. J. (1993). *An Introduction to the Bootstrap*. New York, NY: Chapman & Hall.

Endler, J. A., and Mappes, J. (2017). The current and future state of animal coloration research. *Philos. Trans. R. Soc. B Biol. Sci.* 372:20160352. doi: 10.1098/rstb.2016.0352

Faraway, J. J. (2006). *Extending the Linear Model With R*. Boca Raton, FL: Chapman and Hall/CRC.

Fields, L., Reeve, K. F., Adams, B. J., and Verhave, T. (1991). Stimulus generalization and equivalence classes: a model for natural categories. *J. Exp. Anal. Behav.* 55, 305–312. doi: 10.1901/jeab.1991.55-305

Garcia, J. E., Shrestha, M., and Dyer, A. G. (2018). Flower signal variability overwhelms receptor-noise and requires plastic color learning in bees. *Behav. Ecol.* 29, 1286–1297. doi: 10.1093/beheco/ary127

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path to color discrimination is S-shaped: behaviour determines the interpretation of color models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Genest, C., and Favre, A.-C. (2007). Everything you always wanted to know about copula modeling but were afraid to ask. *J. Hydrol. Eng.* 12, 347–368. doi: 10.1061/(ASCE)1084-0699(2007)12:4(347)

Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi: 10.1007/s00114-004-0530-z

Gray, M., Stansberry, M. J., Lynn, J. S., Williams, C. F., White, T. E., and Whitney, K. D. (2018). Consistent shifts in pollinator-relevant floral coloration along rocky mountain elevation gradients. *J. Ecol.* 106, 1910–1924. doi: 10.1111/1365-2745.12948

Hall, P., and Hart, J. D. (1990). Bootstrap test for difference between means in nonparametric regression. *J. Am. Stat. Assoc.* 85, 1039–1049. doi: 10.1080/01621459.1990.10474974

Hannah, L., Dyer, A. G., Garcia, J. E., Dorin, A., and Burd, M. (2019). Psychophysics of the hoverfly: categorical or continuous color discrimination? *Curr. Zool.* 65, 483–492. doi: 10.1093/cz/zoz008

Hart, N. S., and Hunt, D. M. (2007). Avian visual pigments: characteristics, spectral tuning, and evolution. *Am. Nat.* 169, S7–S26. doi: 10.1086/510141

Hatfield, G. (2003). "Objectivity and subjectivity revisited: color as a psychobiological property," in *Colour Perception: Mind and the Physical World*, eds R. Mausfeld and D. Heyer (New York, NY: Oxford University Press), 187–204. doi: 10.1093/acprof:oso/9780198505006.003.0006

Horridge, A. (2009). Generalization in visual recognition by the honeybee (*Apis mellifera*). A review and explanation. *J. Insect Physiol.* 55, 499–511. doi: 10.1016/j.jinsphys.2009.03.006

Howard, S. R., Shrestha, M., Schramme, J., Garcia, J. E., Avarguès-Weber, A., Greentree, A. D., et al. (2018). Honeybees prefer novel insect-pollinated flower shapes over bird-pollinated flower shapes. *Curr. Zool.* 65, 457–465. doi: 10.1093/cz/zoy095

Jersáková, J., Johnson, S. D., and Kindlmann, P. (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biol. Rev.* 81, 219–235. doi: 10.1017/S1464793105006986

Jersáková, J., Jürgens, A., Šmilauer, P., and Johnson, S. D. (2012). The evolution of floral mimicry: identifying traits that visually attract pollinators. *Funct. Ecol.* 26, 1381–1389. doi: 10.1111/j.1365-2435.2012.02059.x

Jersáková, J., Spaethe, J., Streinzer, M., Neumayer, J., Paulus, H., Dötterl, S., et al. (2016). Does *Traunsteinera globosa* (the globe orchid) dupe its pollinators through generalized food deception or mimicry? *Bot. J. Linn. Soc.* 180, 269–294. doi: 10.1111/boj.12364

Johnson, R. A., and Wichern, D. W. (2007). *Applied Multivariate Statistical Analysis, 6th Edn*. Upper Saddle, NJ: Pearson Prentince Hall.

Johnson, S., and Bytebier, B. (2015). *Orchids of South Africa–A field guide*. Cape Town: Struik Nature.

Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T., et al. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Opt. Soc. Am.* 54:1031. doi: 10.1364/JOSA.54.001031

Kagawa, K., and Takimoto, G. (2016). Inaccurate color discrimination by pollinators promotes evolution of discrete color polymorphism in food-deceptive flowers. *Am. Nat.* 187, 194–204. doi: 10.1086/684433

Kelber, A. (2010). What a hawkmoth remembers after hibernation depends on innate preferences and conditioning situation. *Behav. Ecol.* 21, 1093–1097. doi: 10.1093/beheco/arq115

Kelber, A., Vorobyev, M., and Osorio, D. (2003). Animal color vision–behavioural test and physiological concepts. *Biol. Rev.* 78, 81–118. doi: 10.1017/S1464793102005985

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., et al. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* 185, 705–724. doi: 10.1086/681021

Kojadinovic, I., and Yan, J. (2010). Modeling multivariate distributions with continuous margins using the copula R package. *J. Stat. Softw.* 34, 1–20. doi: 10.18637/jss.v034.i09

Kunze, J., and Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav. Ecol.* 12, 447–456. doi: 10.1093/beheco/12.4.447

Leonard, A. S., Dornhaus, A., and Papaj, D. R. (2011). Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J. Exp. Biol.* 214, 113–121. doi: 10.1242/jeb.047407

Lichtenberg, E. M., Heiling, J. M., Bronstein, J. L., and Barker, J. L. (2020). Noisy communities and signal detection: why do foragers visit rewardless flowers? *Philos. Trans. R. Soc. B Biol. Sci.* 375:20190486. doi: 10.1098/rstb.2019.0486

Livingstone, M., and Hubel, D. (1988). Segregation of form, color, movement, and depth: anatomy, physiology, and perception. *Science* 240, 740–749. doi: 10.1126/science.3283936

Nagler, T., Schepsmeier, U., Stoeber, J., Brechmann, E. C., Graeler, B., and Erhardt, T. (2019). *VineCopula: Statistical Inference of Vine Copulas*. R package version 2.3.0.

Nassi, J. J., and Callaway, E. M. (2009). Parallel processing strategies of the primate visual system. *Nat. Rev. Neurosci.* 10, 360–372. doi: 10.1038/nrn2619

Neumeyer, C. (1980). Simultaneous color contrast in the honeybee. *J. Comp. Physiol. A* 139, 165–176. doi: 10.1007/BF00657079

Newhall, S. M., Burnham, R. W., and Clark, J. R. (1957). Comparison of successive with simultaneous color matching. *J. Opt. Soc. Am.* 47, 43–54. doi: 10.1364/JOSA.47.000043

Paine, K. C., White, T. E., and Whitney, K. D. (2019). Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints? *Evol. Ecol.* 33, 461–479. doi: 10.1007/s10682-019-09991-2

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based color vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Peter, C. I., and Johnson, S. D. (2008). Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89, 1583–1595. doi: 10.1890/07-1098.1

Phillips, R. D., Peakall, R., van der Niet, T., and Johnson, S. D. (2020). Niche perspectives on plant–pollinator interactions. *Trends Plant Sci.* 25, 779–793. doi: 10.1016/j.tplants.2020.03.009

Pinheiro, J. C., and Bates, D. M. (2000). *Mixed-Effects Models in S and S-PLUS*. New York, NY: Springer New York.

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Renoult, J. P., Kelber, A., and Schaefer, H. M. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Reser, D. H., Wijesekara Witharanage, R., Rosa, M. G. P., and Dyer, A. G. (2012). Honeybees (*Apis mellifera*) learn color discriminations via differential conditioning independent of long wavelength (green) photoreceptor modulation. *PLoS ONE* 7:e48577. doi: 10.1371/journal.pone.0048577

Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower color preferences but flowers with similar colors do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103

Rolfe, R. A. (1913). Eulophia zeyheriana Sond. *Flora Capensis* 5:3.

Scaccabarozzi, D., Cozzolino, S., Guzzetti, L., Galimberti, A., Milne, L., Dixon, K. W., et al. (2018). Masquerading as pea plants: behavioural and morphological evidence for mimicry of multiple models in an Australian orchid. *Ann. Bot.* 122, 1061–1073. doi: 10.1093/aob/mcy166

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower color and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019). Floral color structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Shrödinger, E. (1970). "Outline of a theory of color measurement for daylight vision," in *Sources Color Science*, ed D. L. MacAdam (Cambridge, MA: MIT Press), 134–182.

Smith, S. D. (2014). Quantifying color variation: improved formulas for calculating hue with segment classification. *Appl. Plant Sci.* 2:1300088. doi: 10.3732/apps.1300088

Sommerlandt, F. M., Spaethe, J., Rössler, W., and Dyer, A. G. (2016). Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS ONE* 11:e0164386. doi: 10.1371/journal.pone.0164386

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Steiner, K. E. (1998). The evolution of beetle pollination in a South African orchid. *Am. J. Bot.* 85, 1180–1193. doi: 10.2307/2446351

Stoddard, M. C., Eyster, H. N., Hogan, B. G., Morris, D. H., Soucy, E. R., and Inouye, D. W. (2020). Wild hummingbirds discriminate nonspectral colors. *Proc. Natl. Acad. Sci. U.S.A.* 117, 15112–15122. doi: 10.1073/pnas.1919377117

Violle, C., Navas, M.-L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., et al. (2007). Let the concept of trait be functional! *Oikos* 116, 882–892. doi: 10.1111/j.0030-1299.2007.15559.x

von Helversen, O. (1972). "The relationship between difference in stimuli and choice frequency in training experiments with the honeybee," in *Information Processing in the Visual Systems of Anthropods, Chapter 9*, ed R. Wehner (Berlin; Heidelberg: Springer Berlin Heidelberg), 323–334. doi: 10.1007/978-3-642-65477-0_46

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of color thresholds. *Proc. R. Soc. Lond. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302

Welsford, M. R., and Johnson, S. D. (2012). Solitary and social bees as pollinators of Wahlenbergia (Campanulaceae): single-visit effectiveness, overnight sheltering and responses to flower color. *Arthropod. Plant. Interact.* 6, 1–14. doi: 10.1007/s11829-011-9149-0

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Reproductive Assurance Maintains Red-Flowered Plants of *Lysimachia arvensis* in Mediterranean Populations Despite Inbreeding Depression

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Flower color polymorphism, an infrequent but phylogenetically widespread condition in plants, is captivating because it can only be maintained under a few selective regimes but also because it can drive intra-morph assortative mating and promote speciation. *Lysimachia arvensis* is a polymorphic species with red or blue flowered morphs. In polymorphic populations, which are mostly Mediterranean, pollinators prefer blue-flowered plants to the red ones, and abiotic factors also favors blue-flowered plants. We hypothesize that the red morph is maintained in Mediterranean areas due to its selfing capacity. We assessed inbreeding depression in both color morphs in two Mediterranean populations and genetic diversity was studied via SSR microsatellites in 20 natural populations. Results showed that only 44–47% of selfed progeny of the red plants reached reproduction while about 72–91% of blue morph progeny did it. Between-morph genetic differentiation was high and the red morph had a lower genetic diversity and a higher inbreeding coefficient, mainly in the Mediterranean. Results suggest that selfing maintaining the red morph in Mediterranean areas despite its inbreeding depression. In addition, genetic differentiation between morphs suggests a low gene flow between them, suggesting reproductive isolation.

Keywords: *Anagallis*, gene flow, speciation, flower color polymorphism, inbreeding depression, phenology, selfing

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INTRODUCTION

Flower color polymorphism was first defined by Ford (1945) and later by Huxley (1955) as the presence of at least two genetically-determined color morphs within a single interbreeding population, the rarest of which is too frequent to be solely the result of recurrent mutation. Flower color polymorphism is a phylogenetically widespread trait in plants, but is relatively infrequent (Whitney, 2005; Rausher, 2008; Narbona et al., 2018). When a color polymorphism arises, both biotic and abiotic factors can influence the fitness of different morphs. On the one hand, floral morphs can show differential tolerance to abiotic factors (Warren and Mackenzie, 2001;

Arista et al., 2013) due to the association between some floral pigments and protective flavonoids, and be selected either for or against in different habitats. This could eventually result in a segregation of color morphs into different monomorphic populations according to environmental factors, which would lead to the loss of color polymorphism as defined above, although genetic variation remains in the species. However, selection regime could vary due to temporal variation in environmental conditions at the population level and it could thus maintain color polymorphism (Kawecki and Ebert, 2004).

On the other hand, biotic agents of selection such as herbivores (Strauss and Whittall, 2006; Sobral et al., 2015) or pollinators (Meléndez-Ackerman et al., 1997; Jones and Reithel, 2001) can show a preference for a particular morph, thereby being responsible for its lower or higher fitness, respectively. Pollinators are among the most important biotic factors involved in flower color selection (Fenster et al., 2004; Whibley et al., 2006; Schiestl and Johnson, 2013). They usually discriminate between colors and can show preferences for a particular color morph causing directional selection (Waser and Price, 1983; Jones and Reithel, 2001; Ortiz et al., 2015). In polymorphic populations, the less-visited morph could suffer a fitness reduction leading to negative directional selection that, along with genetic drift, could eventually result in the evolution of monomorphic populations (Gigord et al., 2001; Jones and Reithel, 2001), although diverse selective factors and neutral processes could counterbalance that selection, thus maintaining polymorphism (Irwin and Strauss, 2005; Leimar, 2005). For example, negative frequency-dependent selection can be responsible for the maintenance of color polymorphism; this has been found in some rewardless polymorphic species in which pollinators alternate visits between color morphs when they do not find rewards (Gigord et al., 2001; Kagawa and Takimoto, 2016). Temporal variations in pollinator visitation may change the strength and direction of selection, thus generating a balancing selection regime that could maintain color polymorphism (Subramaniam and Rausher, 2000; Schemske and Bierzychudek, 2001). The maintenance of color polymorphism by pollinators can also occur under divergent selection when different kinds of visitors preferably forage on distinct color morphs (Medel et al., 2003).

Some plants have the capacity to produce seeds by autonomous selfing when pollinators are scarce, thereby showing a mixed mating system. This capacity allows plant reproduction when opportunities for outcrossing are reduced and it is a form of reproductive assurance (Holsinger, 2000), as it occurs when pollinator visitation is low. Plants with reproductive assurance capacity via autonomous selfing can be independent of pollinators and can be maintained in populations, at least for some time (Takebayashi and Morrell, 2001; Charlesworth, 2006). In the short term, the potential benefits of selfing may be counteracted by inbreeding depression, that is, fitness reduction of selfed progeny in relation to that of outcrossed progeny (Igic and Busch, 2013). Nevertheless, in plants with repeated selfing, purging effects may eventually lead to decreased inbreeding depression (Lande and Schemske, 1985). However, mixed-mating taxa have inbreeding depression rates as high as those for outcrossing taxa, indicating that allele purging does not always occur (Busch and Delph, 2011). Breeding system has a high influence on gene diversity, and outcrossing species tend to be more diverse genetically and has less genetic differentiation among their populations (Hamrick and Godt, 1996). In some plants with mixed-mating systems, high levels of inbreeding depression hinder the recruitment of selfed progeny, thus recruited progeny comes mainly from outcrossing and the genetic diversity in the populations is maintained (Winn et al., 2011). In contrast, in other plants, reproductive assurance benefits override the inbreeding depression detriment and plants of selfed origin are recruited, which in the long term leads to a reduction in genetic diversity of populations (Glémin et al., 2006). Nonetheless, in conditions of pollen limitation, the reproductive assurance benefits of selfing could be selected (Schoen and Brown, 1991; Arista et al., 2017) and it can be an important mechanism in maintaining polymorphisms at least in the short term (Narbona et al., 2018).

To the best of our knowledge, the role of selfing as a factor maintaining flower color polymorphism has been described in *Ipomoea purpurea* (Rausher et al., 1993; Fry and Rausher, 1997; Subramaniam and Rausher, 2000) and *Boechera stricta* (Vaidya et al., 2018). In *I. purpurea*, color polymorphism is maintained by a combination of negative frequent selection by pollinators and autonomous selfing. When insects stop visiting the white phenotype because its frequency becomes very low, these plants produce seeds through automatic self-pollination, which increases their frequency in the next generation (Subramaniam and Rausher, 2000). In that situation the maintenance of flower color polymorphism is probably temporal as both pollinator behavior and selfing contribute to assortative mating between morphs (Brown and Clegg, 1984; Nosil et al., 2009; Servedio et al., 2011) and could initiate a speciation process (Shivanna, 2015).

Lysimachia arvensis is an annual species described as polymorphic in flower color, with red- or blue-flowered plants that show a geographical pattern of distribution associated to abiotic factors. The blue-flowered plants perform better in dry areas as those that characterize the Mediterranean Basin. In Mediterranean areas, pure blue or blue-biased populations are the most frequent. In contrast, in Atlantic or temperate areas of Europe, where climate is wetter, pure red populations are the norm and the scarce polymorphic populations are strongly red-biased (Arista et al., 2013). *Halictus* and *Lasioglossum* bees, the main pollinators of both color morphs, show a strong and consistent preference for blue-flowered plants in Mediterranean populations (Ortiz et al., 2015; Jiménez-López et al., 2020a), where directional selection gives rise to higher fitness of the blue morph relative to the red morph (Ortiz et al., 2015). No information about pollinator visitation in non-Mediterranean areas exists. Flowers of both colors show herkogamy (Jiménez-López et al., 2019a), but they open and close during 3 days and can self-pollinate during their lifespan, allowing reproductive assurance if outcross pollination fails.

Despite the fact that both abiotic and biotic factors negatively select for the red-flowered plants in the Mediterranean populations, they are maintained in low frequencies over years. Here we study the role of selfing capacity in maintaining red-flowered plants of *Lysimachia arvensis* in Mediterranean

populations. We hypothesize that selfing confers reproductive assurance to the red-flowered plants when pollinator visitation is low, thus maintaining them in Mediterranean populations. Alternatively, their maintenance could be driven by gene flow from temperate areas although the small population sizes and the lack of seed dispersal mechanism hardly support this possibility. If selfing is maintaining red-flowered plants in Mediterranean populations, inbreeding depression should be low enough to allow plant recruitment and the genetic diversity of the red morph should be low in those populations. Thus, we first experimentally analyzed the impact of inbreeding depression through the whole life cycle in two polymorphic Mediterranean populations. Secondly, we characterized the genetic variation and genetic distances of both color morphs in 20 natural populations occurring in Mediterranean and non-Mediterranean areas. If the red morph largely reproduces by selfing in the Mediterranean, this fact could contribute to its reproductive isolation and ecological divergence from the blue morph, and one could expect a genetic divergence as incipient species.

MATERIALS AND METHODS

Study Species

Lysimachia arvensis (L.) U. Manns & Anderb. (former *Anagallis arvensis* L.) is an annual species that inhabits cultivated fields, wastelands and coastal sands (Ferguson, 1972), and is native to Europe and the Mediterranean Basin (Pujadas, 1997). The species is tetraploid and indirect evidences suggest a homopolyploid origin (Monein et al., 2003; Talavera et al., 2020). It is a self-compatible forb that offers only pollen as a reward to pollinators. The two color morphs differ in anthocyanin composition, with malvidine and pelargonidine being mainly responsible for the blue and red coloration, respectively (Harborne, 1968; Ishikura, 1981). Flower color is genetically determined and hand crosses between color morphs originates a homogeneous offspring with salmon colored flowers, which are infrequent in natural populations (Jiménez-López et al., 2020a,b). Thus, although current taxonomy recognizes both color morphs as the same species (Pujadas, 1997; Manns and Anderberg, 2009) it seems likely that they belong to diverging morphs and with scarce reproductive events between them.

In the Mediterranean, where the blue morph is much more frequent, the climate is typically sunnier, hotter and dryer than in the Atlantic or temperate European areas, where the red morph is widely distributed. Water stress is rare in those Atlantic and temperate areas, but it is very frequent in the Mediterranean where there are both an extended aestival drought and other shorter water stress periods during the wet season due to erratic rains. Population sizes are usually less than 100 plants. In the Mediterranean, flowers are visited by small solitary bees that build their nests in the soil near the plants. Bees show a strong preference for blue flowers probably as consequence of their higher visual contrast with the background (Ortiz et al., 2015). In experimental studies carried out in different years and areas, wild bees visit the blue flowers at a higher rate than expected based on their frequency (Ortiz et al., 2015; Jiménez-López et al., 2020a; **Supplementary Figure S1**). The fruit is a capsule and the seeds are dispersed by gravity mainly under the mother plants. Seeds of both morphs germinate during the first or second year after dispersal.

Inbreeding Depression Throughout Life Cycle

Inbreeding depression (ID) at different stages of the life cycle was studied for both flower color morphs under natural field conditions. Given that inbreeding depression depends on the context, we selected two Mediterranean sites where both morphs co-occur: Dos Hermanas (70% blue: 30% red) and Sevilla (60% blue: 40% red). In the studied year, precipitation was 626 mm and mean temperature 19°C; those records fit mean climatic conditions of the last twenty years in these areas where mean precipitation was 576 mm and mean temperature 19.1°C. Both sites consist mainly of herbaceous communities on wastelands around orchards. Seeds from red- and blue-flowered plants were collected from the natural populations, germinated and grown in glasshouses. Among the resulting plants, 16 well-developed individuals were selected and hand self- and cross-pollinations were carried out in each plant to obtain progeny families. In both morphs, selfed seeds were obtained by applying self-pollen to the stigmas of recently opened flowers but without emasculation. Outcrossing seeds were obtained by hand-pollination; recently opened flowers were emasculated and pollen from a different plant of the same color and population was applied to the stigmas. After hand-pollination all the flowers were bagged during their life span to avoid that pollen from unknown sources could reach the flowers. Fruit-set in hand-pollinated flowers was 100%, and the number of seeds was recorded in 40–60 fruits of each cross type and color morph (hereafter seed production of mother plants). A total of 1538 selfed (774 blue and 766 red) and 1507 outcrossed (744 blue and 765 red) seeds were sown, 1517 seeds were from blue plants and 1530 from red ones. In the two natural sites, seeds were placed in individual cardboard pots buried in the ground and each potted seed was treated as an independent experimental unit. Sowing was carried out at the beginning of November, and potted plants were harvested at the end of May. During the growth cycle pots were checked every fortnight, and time from sowing to germination (hereafter time to germination), final seed germination, seedling survival up to reproductive age, time from germination to flowering (hereafter time to flowering) and seed production after free pollination (hereafter seed production of progeny) were recorded for each plant (505 plants; 241 blue and 264 red). Seed production of progeny was estimated as the mean number of seeds in two ripe fruits per plant.

Inbreeding depression was estimated as: $\delta = (W_o - W_s)/W_{max}$ where δ is the inbreeding depression coefficient, $\mathbf{W}_o$ is fitness after outcrossing, $\mathbf{W}_s$ is fitness after selfing, and $\mathbf{W}_{max}$ is the maximum fitness (W_o or W_s). This expression, proposed by Sletvold et al. (2012) or Delmas et al. (2014), derives and is equivalent to that from Ågren and Schemske (1993). The inbreeding depression coefficient ranges between 1 and −1; if $W_o > W_s$, δ values are positive and inbreeding depression exists, while in $W_i > W_o$,

δ values are negative and outbreeding depression occurs (Brys and Jacquemyn, 2016). Inbreeding depression coefficients for each color morph and site were calculated separately at different life stages (partial inbreeding depression coefficients) and then, cumulative inbreeding depression coefficients were also calculated. Partial inbreeding depression coefficients were calculated at each of the following life stages: seed production of mother plants, total seed viability, seedling survival and seed production of progeny. To avoid bias in the assessment of inbreeding depression from germination data due to seed dormancy, a subset of selfed and outcrossed seeds was sown in Petri dishes and placed in a germination chamber. Non-germinated seeds (143 self red, 154 cross red, 123 self blue and 164 cross blue) were placed in a 100 μl solution of tetrazolium 0.11% to determine their viability (Glenner, 1990). Data regarding germination and viability of non-germinated seeds was then considered together, as total seed viability, to calculate inbreeding depression at that stage. Fruit-set was 100% in all the cases and a partial inbreeding depression coefficient at that stage was thus not considered. Cumulative inbreeding depression coefficients were calculated from cumulative fitness values for each cross type and is presented as a proportion. Values of fitness at each life-stage were relativized to the maximum at this stage (W_o or W_s); cumulative fitness for each cross type was then estimated by multiplying relative fitness values at the four life-stages considered (seed production of mother plants, total seed viability, seedling survival and seed production of progeny). When partial inbreeding depression at one life-stage was not significant (zero encompassed by confidence interval), relative fitness of that stage was not included in calculating cumulative inbreeding depression (Dart and Eckert, 2013).

Populations Sampled and Molecular Analyses

Nuclear microsatellite markers were used to assess how genetic variation is structured among and within populations and floral color morphs of *L. arvensis*. To this end, 20 natural populations were sampled (**Supplementary Table S1** and **Supplementary Figure S2**): 14 from the Mediterranean Basin and six from Non-Mediterranean areas. Of these, 11 were polymorphic and nine pure (four blue and five red). Given the evidences that indicate that blue and red-flowered plants are different morphs, polymorphic populations were considered as composed by two subpopulations, one for each morph. Each population was categorized as small (<50 plants), medium (50–100) or large (>100) based on its size (**Supplementary Table S1**). Six plants of each color morph were sampled in polymorphic populations and ten in pure populations. An analysis using genetic diversity accumulation curves (Kamvar et al., 2014, 2015) of multilocus genotypes indicated that a sample of 5–6 plants per population adequately describes the genetic diversity of these populations (**Supplementary Figure S3**). Leaves of these plants were dried in silica gel and stored until molecular analyses were performed. Total genomic DNA was extracted from dry leaf tissue with a plant extraction kit (Invisorb Vegetal DNA Kit HTS 96, Invitek, Berlin, Germany) following the supplier's instructions. The average DNA concentration was estimated photometrically using a NanoDrop DS-11 Spectrophotometer (De,Novix).

A total of 203 individuals out of 222 were correctly sequenced and analyzed at nine microsallite loci (*Lys*11, *Lys*12, *Lys*16, *Lys*28, *Lys*29, *Lys*30, *Lys*31, *Lys*32 and *Lys*33) previously characterized and available for *Lysimachia arvensis* (Jiménez-López et al., 2015). PCR products produced clear amplifications of the expected size on agarose gels. The amplification products were separated by capillary gel electrophoresis on an automated sequencer (3730 DNA Analyser, PE Applied Biosystems, Foster City, CA, United States) with an internal size standard (GeneScan 500 LIZ, Applied Biosystems) at STABVIDA Lda. (Oeiras, Portugal). SSR fluorescence patterns were visualized with GeneMarker 1.9 (SoftGenetics, State College, PA, United States) for manual scoring of fragments after normalization of the profiles. A fluorescence threshold set at 100 relative fluorescent units was applied to validate the peaks that exceeded the fluorescence intensity of this threshold.

Gene diversity was estimated separately, for blue vs. red morphs and for Mediterranean vs. non-Mediterranean areas. Gene diversity was calculated as expected heterozygosity (He) with GENODIVE, version 2.0b25 (Meirmans and Van Tienderen, 2004), and observed heterozygosity (Ho) with ATETRA version 1.2 (Van Puyvelde et al., 2010). Allele number (A), and inbreeding coefficient (G_{IS}) were also calculated with GENODIVE 2.0b25, assuming infinite alleles and corrected for unknown allele dosage. Linkage disequilibrium (LD) was calculated with genetics R package *poppr* (Warnes and Leisch, 2005; Kamvar et al., 2014), and null allele frequency (No) was estimated by POLYSAT (Clark and Jasieniuk, 2011). The disadvantage of allele dosage in polyploids was minimized by the method of Bruvo et al. (2004), implemented in POLYSAT, which consider the distances between the microsatellite alleles without knowing the number of copies of the alleles. Afterward, the *combn* and *permn* R functions were used to match all possible combinations and to find the smallest sum of geometrically transformed distances between alleles (see Clark and Jasieniuk, 2011 for more details).

Based on the allelic differences observed between blue- and red-flowered plants, we performed fast cluster analysis. On the one hand, a principal coordinate analysis (PCoA) among individuals of all studied populations was constructed. First the matrix of Jaccard similarities among individuals was calculated with the function "vegdist" of the vegan R package (Oksanen et al., 2013) and then the PCoA was calculated using the function "pco" of the ecodist R package (Goslee and Urban, 2007) and plotted in R software. On the other hand, the overall population genetic structure was explored using model-based Bayesian assignment running STRUCTURE 2.3.4 (Pritchard, 2010). Analyses were based on an admixture ancestry model with correlated allele frequencies, for a range of K genetic clusters from 1 to 18, with 10 replicates for each K. The analyses were performed with a burn-in period of 100000 and a run length of the Monte Carlo Markov Chain (MCMC) of 10^6 iterations. Following Evanno et al., 2005, the most likely number of genetic clusters (K) was determined according to the DK, using STRUCTURE Harvester (Earl and vonHoldt, 2012).

Furthermore, to discard the possibility that among-population gene flow maintains color polymorphism in the Mediterranean, isolation-by-distance between populations was investigated by computing the correlation between the matrix of pair-wise population genetic distance obtained by SSR (ΦPT) and the matrix of geographical distances, by applying the Mantel test (10000 permutations). Mantel test was performed in two ways, considering the two morphs together and for each morph separately. Given that *L. arvensis* lacks dispersal mechanism, gene flow is constrained by geographical distance and it would be much more likely to occur between neighboring populations, following a stepping-stone model.

Statistical Analyses

In exploring the possibility of inbreeding depression, generalized linear mixed model (GLMM) analysis were performed to test for the effects of site, cross type and color (fixed factors), and the two-way interaction color by cross type and the three-way interaction, on seed production of mother plants, viability of non-germinated seeds, final seed germination, seedling survival and seed production of progeny (dependent variables). Plant was included as a random variable to incorporate random effects. GLMMs were carried out with different link functions and error distributions, depending on the type of response variable modeled. Binomial distribution of errors and logit link function were used to analyze germination, viability and survival. Binomial negative distribution with log link function was used to analyze time to germination, and normal distribution to analyze time to flowering and seed production. All these analyses were carried out using the GLMM module of SPSS (IBM SPSS Statistic 23, 2015, United States) with Type III tests. When GLMMs showed significant differences, the means of treatments were compared using t-tests based on standard errors calculated from the specific model. Also 95% Confidence intervals (95% CI) were estimated by bootstrap resampling method (10000 replicates).

Time to germination and time to flowering between self- and outcross progeny were analyzed by means of Time-to-event analyses (survival analyses). We used Kaplan–Meier curves combined with the log-rank test for difference. We used the "1 minus Kaplan–Meier" curves to show the proportion of planted seeds that had germinated on any given day after planting or the proportion on plants that had flowered on any day after seed germination.

To distribute genetic variation as estimated from SSR markers among and within populations and floral color morphs, multi-locus analyses of molecular variance (AMOVA) were performed using Arlequin (Schneider et al., 2000). These analyses hierarchically partitioned molecular variation into within- and among-population components to estimate genetic structure in the following predefined groups: blue vs. red plants, Mediterranean vs. non-Mediterranean, blue Mediterranean vs. red Mediterranean, blue non-Mediterranean vs. red non-Mediterranean. Permutation tests were used to determine statistical significance (Excoffier et al., 1992). Statistics for the significance (OSx-statistic; Goudet, 1995) across all groups or between pairs of comparison were obtained by 9999 randomizations using GENODIVE 2.0b25. The possible influence of population size on the genetic diversity of morphs (measured as observed heterozygosity) was tested by ANOVA with population size and color as factors.

RESULTS

Inbreeding Depression Throughout Life-Cycle

The number of seeds per capsule obtained after hand-pollination was higher in the red morph than in the blue one, and it was also higher after selfing than after outcrossing but was unaffected by seed origin (**Table 1**). The color-by-treatment interaction was significant (**Table 1**), as only in the blue morph seed production was significantly higher after selfing than after outcrossing (**Figure 1**). Thus, at this first stage of the life cycle,

TABLE 1 | Results of GLMM used to test for the effects of treatment (selfing/outcrossing), color morph (blue/red) and site (Sevilla/Dos Hermanas) on different traits measured in *Lysimachia arvensis*.

Trait	Effect	df1	df2	F/Z	*p*
Seed production of mother plants	Site (S)	1	473	0.000	0.989
	Treatment (T)	1	473	1.304	0.254
	Color (C)	1	473	48.347	**0.000**
	T x C	1	473	26.362	**0.000**
	S x T x C	3	473	0.414	0.743
	Family	15		0.596	0.551
Seed germination	Site (S)	1	3036	0.010	0.921
	Treatment (T)	1	3036	0.032	0.859
	Color (C)	1	3036	12.379	**0.000**
	T x C	1	3036	0.122	0.727
	S x T x C	3	3036	2.186	0.088
	Family	15		1.959	**0.050**
Viability of non-germinated seeds	Site (S)	1	576	0.013	0.910
	Treatment (T)	1	576	84.912	**0.000**
	Color (C)	1	576	0.088	0.767
	T x C	1	576	0.219	0.640
	S x T x C	3	576	0.277	0.842
	Family	15		2.174	0.030
Seedling survival	Site (S)	1	1179	0.005	0.944
	Treatment (T)	1	1179	0.008	0.929
	Color (C)	1	1179	14.506	**0.000**
	T × C	1	1179	1.848	0.174
	S × T × C	3	1179	2.600	0.051
	Family	15		1.051	0.293
Seed production of progeny	Site (S)	1	308	0.008	0.928
	Treatment (T)	1	308	52.709	**0.000**
	Color (C)	1	308	1.181	0.278
	T × C	1	308	4.199	**0.041**
	S × T × C	3	308	1.367	0.253
	Family	15		1.092	0.275

Maternal family was used as a random factor. Significant p-values are indicated in bold. F-value is shown for the fixed effects and Wald Z for the random factor. Significant values are shown in bold.

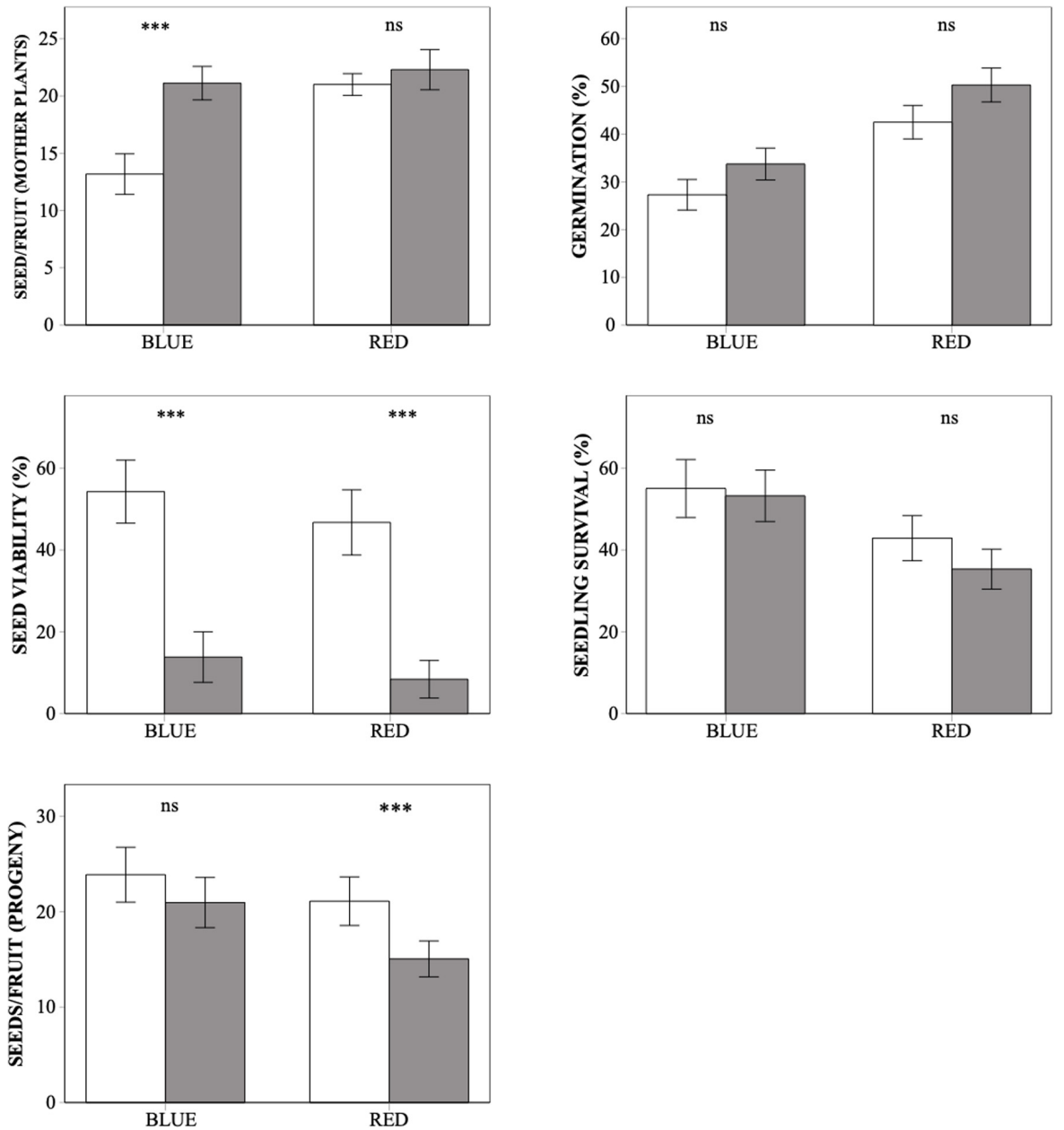


FIGURE 1 | Differences in whole-life fitness components between outcrossed (white bars) and selfed (dark bars) progeny from the blue and red plants of *Lysimachia arvensis* in the Mediterranean. Note that seeds/fruit by mother plants are derived from hand self- and cross-pollination, while seeds/fruit of the progeny come from free pollination of plants resulting from selfing and outcrossing (see section "Materials and Methods"). Means ± SE are shown. In each graph, asterisks indicate significant differences between selfing and outcrossing values for each color morph. ***$p < 0.001$, ns. Not significant.

inbreeding depression coefficient was negative for both color morphs, although it was not significantly different from zero for the red plants (**Table 2**).

Seeds germinated significantly earlier in Sevilla than in Dos Hermanas, in outcrossed than in selfed seeds, and in seeds from the blue than those from the red plants (**Table 3** and **Figure 2**). In both populations, the germination curves followed the same pattern: outcrossed blue seeds germinated earlier, followed by self-blue and outcrossed-red and being the self-red the last to germinate (**Figure 2**). Final seed germination also differed between morphs, but not between sites or treatments (**Table 1**). In general, final germination was higher in seeds from red morph than in blue ones. Seed viability of non-germinated seeds only differed between treatments (**Table 1**); most non-germinated outcrossed seeds were dormant but viable, while only a low proportion of non-germinated selfed seeds were viable (**Figure 1**). Taking into account germination and viability of non-germinated seeds, inbreeding depression coefficient at this stage

TABLE 2 | Estimates of inbreeding depression for plants of the two color morphs of *Lysimachia arvensis* in two natural sites.

	Seed production of mother plants		Total seed viability		Seedling survival		Seed production of progeny		Cumulative	
	Blue	**Red**	**Blue**	**Red**	**Blue**	**Red**	**Blue**	**Red**	**Blue**	**Red**
Sevilla										
Wo	13.37 (11.72, 15.72)	20.51 (19.48, 21.15)	84.25 (80.47, 87.33)	88.50 (85.01, 92.00)	43.93 (34.46, 53.87)	34.02 (29.80, 37.86)	22.67 (19.67, 25.07)	22.19 (19.25, 26.00)	0.41 (0.38, 0.45)	1.00 (0.99, 1.00)
Ws	21.65 (20.49, 22.63)	22.07 (20.59, 24.52)	31.25 (20.67, 40.07)	56.75 (44.74, 68.67)	64.41 (52.17, 77.24)	28.86 (16.41, 43.18)	21.37 (17.13, 25.09)	14.88 (12.60, 16.67)	0.37 (0.24, 0.49)	0.44 (0.31, 0.60)
δ	−0.38 (−0.47, −0.22)	−0.06 (−0.26, 0.04)	0.62 (0.51, 0.80)	0.35 (0.17, 0.51)	−0.32 (−0.39, −0.29)	0.20 (−0.21, 0.51)	0.05 (−0.14, 0.37)	0.32 (0.19, 0.41)	0.09 (−0.16, 0.59)	0.56 (0.32, 0.70)
Dos Hermanas										
Wo	12.99 (11.86, 14.87)	21.50 (20.53, 22.66)	80.00 (76.01, 83.33)	90.00 (89.00, 91.33)	62.48 (60.62, 63.91)	48.61 (38.92, 54.33)	25.07 (21.67, 27.68)	20.01 (18.67, 20.91)	0.63 (0.53, 0.77)	1.00 (0.99, 1.00)
Ws	20.60 (18.54, 22.52)	22.53 (20.77, 24.43)	61.50 (59.59, 63.67)	55.25 (52.00, 58.87)	44.22 (36.84, 52.43)	39.24 (27.74, 52.06)	20.55 (19.35, 21.71)	15.22 (13.23, 17.09)	0.44 (0.37, 0.49)	0.47 (0.39, 0.55)
δ	−0.36 (−0.48, −0.12)	−0.04 (−0.08, 0.34)	0.30 (0.23, 0.39)	0.52 (0.33, 0.77)	0.29 (0.14, 0.40)	0.18 (−0.36, 0.52)	0.16 (0.09, 0.30)	0.23 (0.01, 0.38)	0.28 (0.11, 0.42)	0.53 (0.43, 0.61)

Wo and Ws are fitness measures after outcrossing and selfing, respectively, at different life stages (seed production of mother plants, number per fruit; total seed viability,%; seedling survival,%; and seed production per fruit of progeny) or total for the life cycle (cumulative, proportion); δ is the inbreeding depression coefficient at each of the stages or its cumulative value. 95% Confidence intervals are shown in brackets.

(total seed viability) was high, although it showed contrasting patterns between morphs and sites (**Table 2**).

Seedling survival showed differences between color morphs but not between sites or treatments (**Table 1**). Seedlings from the blue morph showed a significantly higher survival than those from the red morph (**Figure 1**). Inbreeding depression coefficient at this stage was negative for blue plants in Sevilla but positive for the remaining cases, although for the red morph it was not significantly different from zero in any site (**Table 2**).

Flowering time showed significant differences between sites, treatments and morphs (**Table 3**). Flowering occurred earlier in Dos Hermanas than in Sevilla, in outcrossed than in selfed plants, and in the blue morph than in red morph (**Figure 2**). The flowering order being as follows: blue outcrossed plants, red outcrossed plants, blue selfed plants, and red selfed plants (**Figure 3**). These flowering orders appeared in the two sites studied and as a consequence, there were *L. arvensis* plants in flower for almost 5 months.

In free pollination, the number of seeds per fruit of the progeny varied significantly between treatments, but not between sites or color morphs (**Table 1**). In general, outcrossed plants produced a mean of 22.21 seeds while selfed plants produced 18.22 seeds. The interaction color by treatment was also significant, as differences in seed production between treatments were more marked in the red morph than in the blue morph (**Figure 1** and **Table 1**). Inbreeding depression coefficient at this stage was positive in both morphs and sites, but it was not significant in the blue morph in Sevila (**Table 2**).

Cumulative inbreeding depression measures were positive for both morphs in both sites, although for the blue morph in Seville it was not significantly different from zero. Red plants showed a consistent high inbreeding depression cumulative value of more than 0.5 in both sites, whereas in the blue plants it varied from 0.09 in Sevilla to 0.28 in Dos Hermanas (**Table 2**).

TABLE 3 | Results of Kaplan–Meier log rank estimate test for both germination and flowering distributions.

Test	Chi-square	df	*p*-value
Germination			
Site	32.162	1	**0.000**
Treatment	43.85	1	**0.000**
Color	52.688	1	**0.000**
Treatment vs. Color	102.046	3	**0.000**
Flowering			
Site	35.008	1	**0.000**
Treatment	192.238	1	**0.000**
Color	111.760	1	**0.000**
Treatment vs. Color	384.114	3	**0.000**

Significant values are shown in bold.

Properties of Microsatellites

The nine microsatellite loci were successfully genotyped in the 203 individuals of *L. arvensis*. There were cases of deviation from HWE ($P < 0.05$) after Bonferroni correction across populations and loci; the most significant cases were related to negative or high levels of G_{IS}, indicating HWE deviation caused by heterozygote excess or deficiency, respectively. Null allele frequency (No) estimated using POLYSAT resulted in an average frequency of 0.138 in the nine markers ranging from 0 to 0.478. According with *poppr* analysis significant LD was found between pairwise combinations of loci in red populations ($\bar{r}_d$ = 0. 1752, $p < 0.001$; Mediterranean $\bar{r}_d$ = 0.1263, $p < 0.001$ and Non-Mediterranean $\bar{r}_d$ = 0.1673, $p < 0.001$), but not in blue populations ($\bar{r}_d$ = 0. 0185, $p > 0.100$; Mediterranean

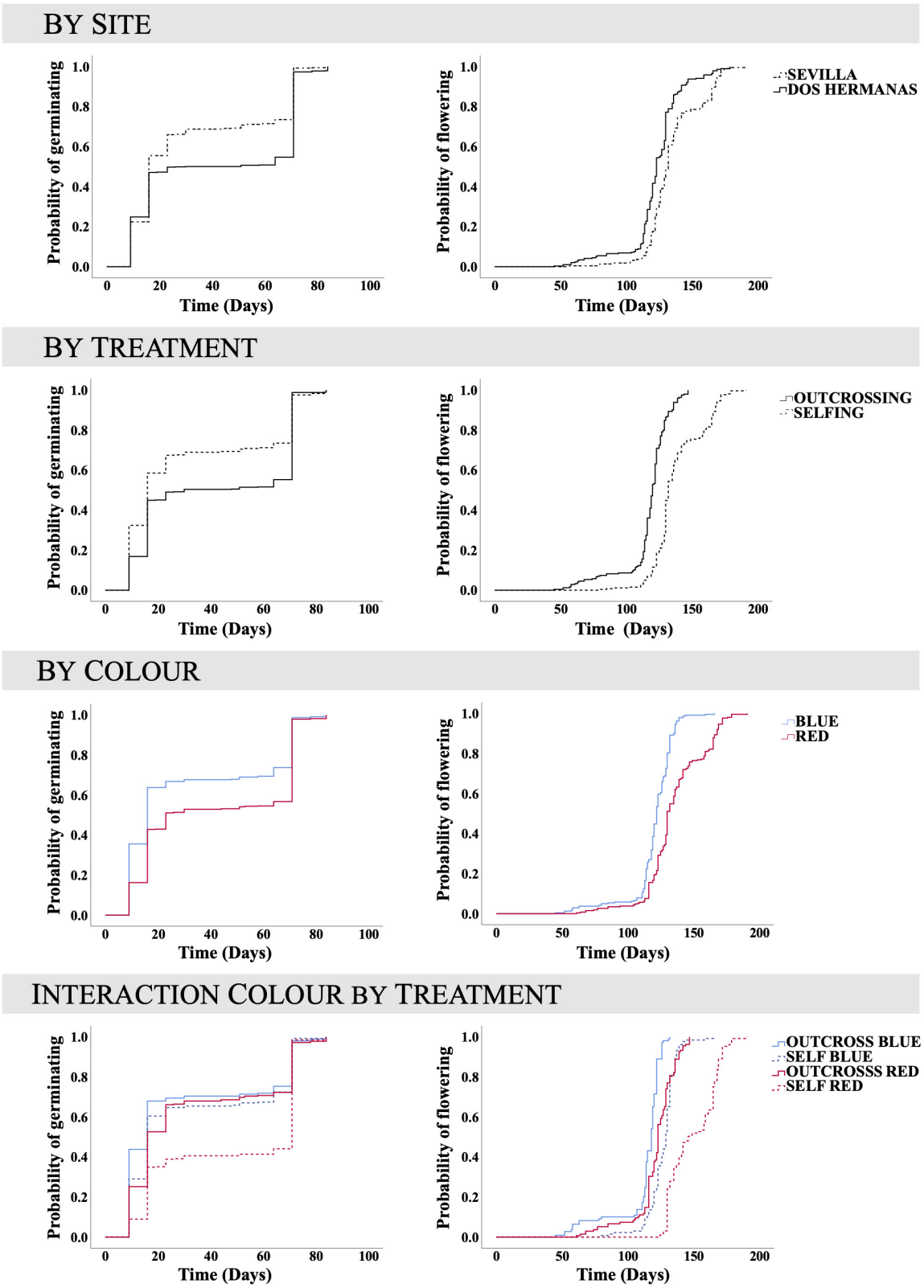


FIGURE 2 | Curves for cumulative germination proportion and cumulative flowering proportion (1 minus the Kaplan–Meier curve) of *Lysimachia arvensis*. Curves by population, treatment (selfing versus outcrossing) and morph (blue versus red) are shown. There was significant effect of these factors on both germination and flowering. The interaction morph by treatment was also significant.

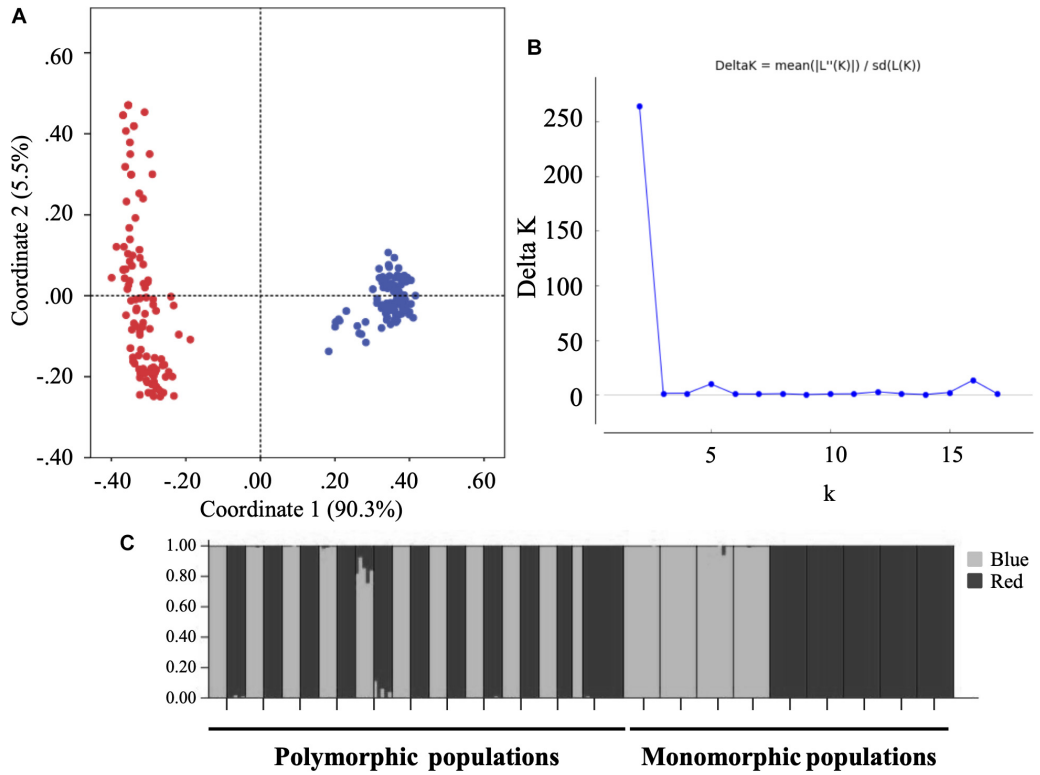


FIGURE 3 | (A) Principal coordinates analysis (PCoA) based on Nei and Li distances at the individual level. **(B)** DK curve for detecting the number of K groups that best fits the data (Evanno et al., 2005). **(C)** Structure at populations level for Bayesian Analysis conducted with STRUCTURE with optimal value, $K = 2$; blue-flowered plants (white bars) and red-flowered plants (black bars).

$\bar{r}_d = 0.02408$ $p > 0.100$ and Non-Mediterranean $\bar{r}_d = 0{,}1189$ $p < 0.010$).

Gene Diversity and Population Structure

In a total of 203 plants and nine SSR analyzed, the total number of alleles was 74 and the mean number of alleles per locus by population and morph ranged from 1.602 to 2.925. In the PCoA conducted at individual level (**Figure 3**), the first two axes explained 90.4 and 5.5% of the total variation, respectively, and separated completely blue-flowered and red-flowered plants. Bayesian clustering with STRUCTURE was consistent with the results of PCoA analyses. Bayesian clustering showed that the greatest informative representation of overall genetic structure was achieved with K = 2 (DK = 264.3196; **Figure 3**). For the 10 replicates of K = 2, the first cluster was very homogeneous and consisted exclusively of blue-flowered individuals while the second cluster comprised only red-flowered individuals (**Figure 3**). Furthermore, the Mantel test indicated no correlation among geographic and gene distances of populations ($r = 0.003$, $p = 0.499$). In general per locus, expected heterozygosity (He) ranged from 0.000 to 0.862, observed heterozygosity (Ho) from 0.000 to 0.900 and the inbreeding coefficient (G_{IS}) from −0.350 to 1.000 (with −0.800 as an outlier value) but most values were from −0.1 to 0.4 (**Supplementary Table S1**). Multilocus estimates per populations showed He ranged from 0.284 to 0.666, Ho ranged from 0.111 to 0.678 and G_{IS} from −0.177 to 0.507 (**Supplementary Table S2**). Significant positive values of G_{IS} were found in some populations for the nine loci, indicating lack of heterozygotes (**Supplementary Tables S1, S2**).

Taking into account, the results of the ordination and Bayesian analyses we compared the genetic parameters between color morphs. The blue morph showed significantly higher observed heterozygosity (Ho) values and lower inbreeding coefficient (G_{IS}) than the red morph (Ho: 0.562 blue vs. 0.415 red, G_{IS}: 0.330 blue vs. 0.618 red; $p < 0.05$ in all cases). However, the expected heterozygosity was similar between morphs (He: 0.657 blue vs. 0.650 red; $p > 0.05$). On the other hand, populations of Mediterranean and non-Mediterranean areas showed no significant differences in observed, expected heterozygosity and G_{IS} (Ho: 0.496 Med. vs. 0.436 Non-Med., $p > 0.05$; He: 0.527 Med- vs. 0.454 Non-Med., $p > 0.05$; G_{IS}: 0.060 Med. vs. 0.040 Non-Med., $p > 0.05$).

In the Mediterranean studied areas the blue morph showed significantly higher observed and expected heterozygosity and lower inbreeding coefficient than the red morph; in contrast, in non-Mediterranean areas the observed and expected heterozygosity were statistically similar between morphs, but the inbreeding coefficient was statistically higher in the blue morph (**Figure 4**). According to the Mantel tests neither blue-flowered populations nor red-flowered populations showed any association between genetic distance and the geographical

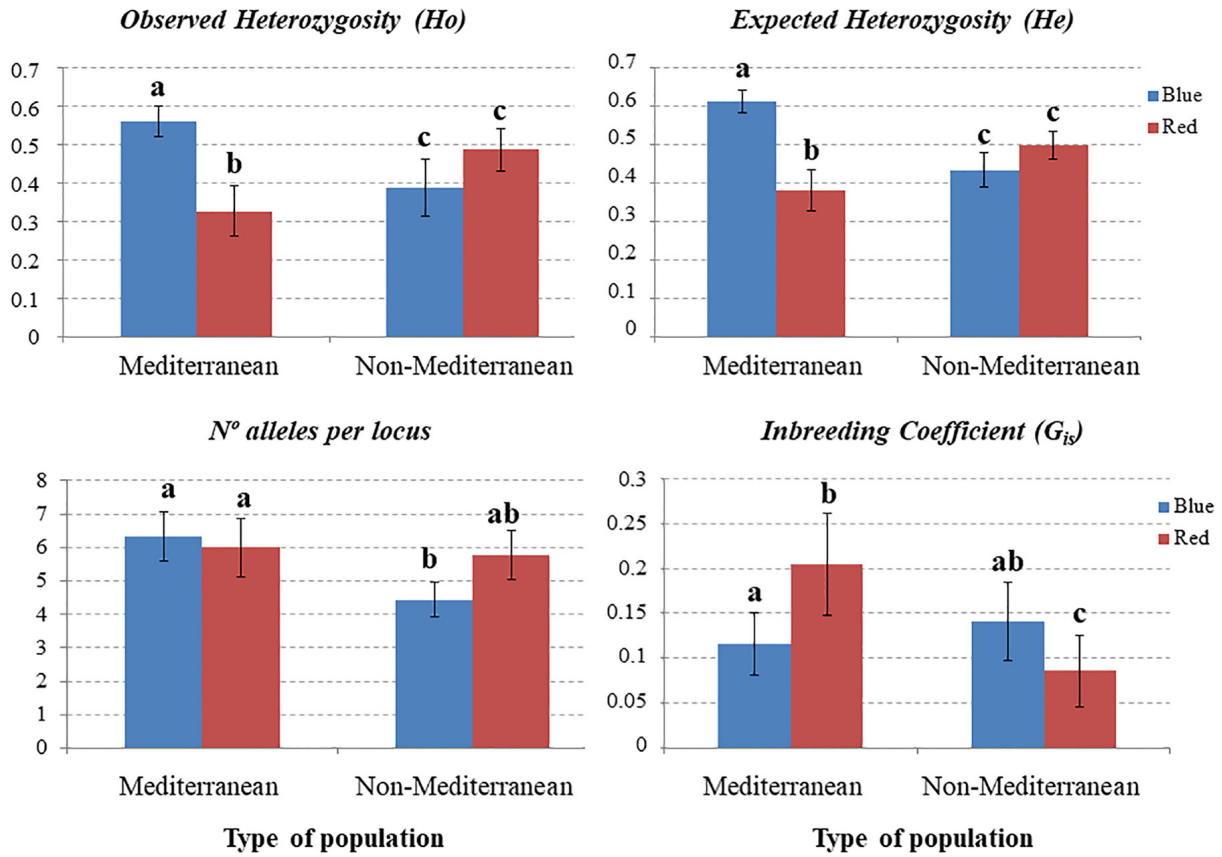


FIGURE 4 | Barplots of gene parameters (mean values and standard error) for blue and red morphs of *Lysimachia arvensis* at nine SSR loci in Mediterranean and non-Mediterranean populations. Within each plot, dataset with the same letter are statistically similar. In all cases, Gis showed deviations from the Hardy-Weinberg equilibrium ($p < 0.05$).

distance between pairs of populations (for the blue morph, $R = 0.145$, $p = 0.226$; for the red morph $R = 0.185$, $p = 0.131$). Therefore, the hypothesis of isolation by distance was rejected for both color morphs. In addition, no significant differences in genetic diversity were observed among populations differing in size, either without considering color morphs ($F_{2,30} = 0.513$, $p = 0.604$) or considering them (blue-flowered plants $F_{2,14} = 1.502$, $p = 0.262$; red-flowered plants $F_{2,15} = 0.021$, $p = 0.979$).

AMOVA consistently demonstrated a significant population structure separating clearly blue and red morphs (**Table 4**). In the Mediterranean, 59.59% of genetic variability was explained by differences in flower color while in non-Mediterranean areas this proportion was slightly lower (52.37%). Plants from Mediterranean areas were also differentiated from those in non-Mediterranean areas, although the proportion of explained variance was low (**Table 4**).

DISCUSSION

Three main results are derived from this study: (1) a higher inbreeding depression rate for the red-flowered plants relative to the blue plants that would render recruitment of red-selfed progeny difficult in natural populations; (2) a lower genetic diversity of the red morph relative to the blue morph, mainly in Mediterranean areas and not related to an isolation by distance pattern, which suggests differences in breeding system between morphs; and (3) a genetic differentiation between morphs that suggests some degree of reproductive isolation.

Marked differences in the fitness of the progeny derived from selfing and outcrossing were found in both color morphs throughout their life cycle. Fruits originated from selfing produced a higher number of seeds than those from outcrossing in both morphs. This result was unexpected, given that seed viability was much higher after outcrossing and a high impact of selfing was found in the remaining steps in the life cycle. Given that the flowers of *L. arvensis* are very small and that emasculation is difficult without damaging the flower, floral manipulation should be considered as a possible cause of decreased seed production in hand-outcrossed flowers. Selfed and outcrossed seeds did not differ in final germination but although outcrossed seeds that did not germinate were viable and remained dormant, ungerminated selfed seeds were dead. This suggests that the seed-banks of *L. arvensis* in the wild should consist of outcrossed seeds from which individuals could be incorporated into the

TABLE 4 | Results of analyses of molecular variance (AMOVA) for nine SSR loci and for different groupings of populations.

	Source of variation	d.f	Sum of squares	Variance components	% Variance explained	Fixation indices	P
Blue vs. Red	Among groups	1	628.363	5.99817	57.19221	FSC: 0.66957	***
	Among populations within groups	29	608.400	3.00610	28.66296	FST: 0.85855	***
	Within populations	172	255.158	1.48347	14.14483	FCT: 0.57192	***
Mediterranean vs. Non-Mediterranean	Among groups	1	66.654	0.29612	3.81415	FSC: 0.80135	***
	Among populations within groups	29	1170.109	5.98413	77.07807	FST: 0.80892	***
	Within populations	172	255.158	1.48347	19.10777	FCT: 0.03814	*
Blue Mediterranean vs. Red Mediterranean	Among groups	1	516.094	6.45105	59.59842	FSC: 0.63192	***
	Among populations within groups	20	414.295	2.76347	25.53052	FST: 0.85129	***
	Within populations	121	209.258	1.60967	14.87107	FCT: 0.59598	***
Blue Non-Mediterranean vs. Red Non-Mediterranean	Among groups	1	116.934	4.64348	52.37656	FSC: 0.74116	***
	Among populations within groups	7	122.786	3.12923	35.29645	FST: 0.87673	***
	Within populations	51	45.900	1.09286	12.32698	FCT: 0.52377	***

*Statistical significance is based on 10100 permutations. *$p < 0.05$, ***$p < 0.001$.*

populations every year. Thus, if dormant outcrossed seeds from the red morph are stored in the seed bank, their germination in successive years could help to polymorphism maintenance, masking the effect of selection on a particular year.

The progeny derived from selfing and outcrossing also showed marked differences in phenology in both morphs. This was an unexpected finding, because differences in phenology according to breeding system are not usually reported in the literature. Non-dormant seeds of *L. arvensis* germinated just after a rainy period giving rise to pulses in which the germination order was: first outcrossed blue, then selfed blue and outcrossed red, and selfed red last. In annuals, the time of germination is the first major developmental transition influencing all posterior life cycle traits (Finch-Savage and Leubner-Metzger, 2006; Manzano-Piedras et al., 2014). Arid environments such as those in the Mediterranean are characterized by limited and variable rainfall that supplies resources in pulses (Chesson et al., 2004). In these environments, quick germination just after rain permits seedlings to develop deep roots to tolerate water scarcity, thereby increasing survival probability (Schenk and Jackson, 2002). As blue seeds germinated earlier than red ones, differences in survival found between colors could be a result of germination differences. Survival differences between morphs in dry environments were found experimentally in a previous work (Arista et al., 2013); thus this study confirms previous findings. In the red morph, differences in the time of germination between selfed and outcrossed seeds were much more marked than in the blue morph; consequently, differences in survival between selfed and outcrossed seedlings were higher.

Flowering phenology was also markedly affected by progeny origin, with blue plants flowering earlier than red ones, and with outcrossed plants flowering earlier than selfed ones. This implies a variation in flowering phenology between color morphs, as previously reported in greenhouse (Arista et al., 2013), although some overlap also occurs. Differences in flowering phenology between morphs could limit pollen flow between them in polymorphic populations, promoting assortative mating within color morphs. Even if color morphs show a temporal overlap in flowering phenology, assortative mating can be much stronger than expected as the chance of mating is reduced (Fox and Kelly, 1993; Fox, 2003). Thus, a difference in flowering phenology acts as a strong prezygotic barrier to gene flow (Martin and Willis, 2007; Botes et al., 2008) and given that prezygotic barriers generally make a greater contribution to reproductive isolation than postzygotic barriers (Lowry et al., 2008; Widmer et al., 2009), flowering phenology could contribute efficiently to morph isolation in *L. arvensis*.

On the other hand, flowering phenology is constrained by both abiotic and biotic factors (Cruz-Neto et al., 2011), and can strongly influence plant reproductive success (Ollerton and Lack, 1998). In annual plants, an early flowering when water is available permits an extended flowering period, and in seasonal climates such as the Mediterranean, this is advantageous as it assures plant reproduction (Rodríguez-Pérez and Traveset, 2016). In contrast, a late flowering increases the risk of drought and limits vegetative growth and fruit production (Giménez-Benavides et al., 2007). In fact, we have found that seed production showed the same pattern as flowering phenology, with higher production in plants flowering earlier (outcrossed blue) and lower in plants flowering later (selfed red). Thus, in the Mediterranean areas studied, the very late flowering of selfed red plants is markedly disadvantageous, strongly limiting production of viable seeds. However, given that the plants studied grew in natural conditions, differences in seed production between morphs could also be a consequence of differential pollinator visitation to them, as has been repeatedly reported in the natural Mediterranean area of *L. arvensis* where the blue morph receive a higher visit rate than the red morph (Ortiz et al., 2015; Jiménez-López et al., 2020a). We have not measured pollen viability of selfed and outcrossed plants; if selfed plants produced less viable pollen, as found in other species (Ellmer and Andersson, 2004; Busch, 2005), their capacity to sire progeny would be also lower.

The red morph consistently had a lower genetic diversity and a higher inbreeding coefficient than the blue morph. The significant LD found in the red morph also suggests a higher selfing rate in this morph (Flint-Garcia et al., 2003). Plant

mating systems have significant effects on genetic diversity (Charlesworth and Wright, 2001), with selfers showing much lower diversity than outcrossers. These differences between selfers and outcrossers are expected to be even more pronounced when both kinds of plants co-occur within populations (Glémin et al., 2006). The strongest differences in inbreeding coefficient were found in the red morph between Mediterranean and non-Mediterranean areas while the blue morph showed similar inbreeding coefficient across areas. This would imply that mating system is context-dependent for the red morph, with a higher selfing degree in the Mediterranean than in non-Mediterranean areas. In contrast, the similar inbreeding coefficient for the blue morph in both areas suggests that mating system does not differ throughout its distribution. Dissimilarity in pollinator visitation to the red-flowered plants in Mediterranean and non-Mediterranean areas could explain these differences. In Mediterranean populations, where the red morph is usually much less frequent due to unfavorable abiotic conditions (Arista et al., 2013), pollinators discriminate against it because they prefer the blue morph (Ortiz et al., 2015; Jiménez-López et al., 2020a) and given its capacity for self-pollination, undervisited flowers can produce seeds by selfing. Long-term consequence of selfing is a decrease in gene diversity (Charlesworth and Wright, 2001) as that found in the red morph in the Mediterranean. Thus, pollen limitation of the red-morph in Mediterranean areas could explain the lower genetic diversity of these populations as compared to those of non-Mediterranean areas; similar situations being found in other taxa (Brito et al., 2016). Alternatively, other historical events may also have caused genetic bottlenecks increasing the genetic drift and decreasing gene diversity in the Mediterranean (Fady and Conord, 2010). However, these processes were not evaluated by this study.

The higher inbreeding coefficient and the low genetic diversity of red plants in the Mediterranean suggest that some selfed progeny is recruited in populations, despite the high values of inbreeding depression found in the field. Differences in fitness between selfed and outcrossed progeny were found in both morphs, although with different intensity. The ranges of inbreeding depression found in *L. arvensis* are in accordance with those of plants with mixed reproductive systems (Winn et al., 2011), ranging from 0.2 to 0.8. Interestingly, inbreeding depression in the blue morph was close to that of selfing species (cumulative δ = 0.09 and 0.28), while red morph inbreeding depression was close to that of outcrossing (cumulative δ = 0.53 and 0.56). According to Winn et al. (2011) if purging is occurring, inbreeding depression of mixed-mating species should be closer to that of selfing species as occurs in the blue morph; this suggests an evolutionary trend toward selfing in this morph. In contrast, the high inbreeding depression of the red morph suggests that allele purging did not occur, which is in accordance with values of inbreeding depression observed in species that typically outcross (Husband and Schemske, 1996). A similar situation has been found in other species with mixed mating system (Voillemot and Pannell, 2017).

The high inbreeding depression rates in the red morph contrasted with both the inbreeding coefficient and its low genetic diversity in polymorphic Mediterranean populations. In the two sites studied the inbreeding depression of the red plants was 0.53–0.56, that mean that only 44–47% of selfed progeny was recruited in populations, while outcross progeny recruited 100%. In fact, in our field experiment selfed plants from the red morph reached reproductive maturity although in much lower proportion than outcrossed plants. Thus, although the impact of inbreeding depression on the fitness of the red plants was markedly high, selfing could be the sole way to ensure reproduction under a pollen limitation scenario. This is particularly important for annual plants that must produce seeds before dying, and even low quality offspring make some contribution to fitness (Charlesworth, 2006). Moreover, in *L. arvensis* selfing occurs when opportunities for outcrossing have passed, thus avoiding an extra cost of pollen and seed discounting. Given that the red morph is negatively selected by both biotic and abiotic factors in the Mediterranean Basin (Arista et al., 2013; Ortiz et al., 2015), the survival of any selfed progeny would provide reproductive assurance (Kalisz et al., 2004), contributing to maintain the red morph. Additionally, we have to stress that inbreeding depression was measured only in two populations in one reproductive cycle. Since the impact of inbreeding depression depends on the context (Cheptou and Donohue, 2010), it is possible that it differs in other years or populations. In any case, since the inbreeding depression values obtained have been high and contrary to our expectations, the possibility of lower inbreeding depression values for the red morph in other populations or years does not invalidate our conclusion.

The analyses of genetic variation in *L. arvensis* showed a strong partitioning of molecular variation between the red and blue morphs in both Mediterranean and non-Mediterranean areas. Genetic differentiation between color morphs strongly suggests that gene flow between them is restricted, being to some extent reproductively isolated. This is also supported by other results found in previous studies and in other previous papers: niche differentiation with the blue-flowered plants more adapted to dry habitats (Arista et al., 2013), differences in flowering phenology found here and in a previous study (Arista et al., 2013) that hinder pollen flow between morphs at least partially, pollinator visitation in polymorphic populations where bees show floral constancy and prefer blue-flowered plants (Ortiz et al., 2015; Jiménez-López et al., 2020a), differences in inbreeding depression and mating system (in this study), and the low frequency of intermediate phenotypes in polymorphic populations (Jiménez-López et al., 2019a; 2020b). These facts clearly indicate a history of gene flow limitation between morphs, suggesting they are different lineages. Flower color polymorphism is a trait that promotes divergent selection (Forsman et al., 2008; McLean and Stuart-Fox, 2014) and is considered a "magic trait" in speciation, that is, a trait "encoded by genes subject to divergent selection that also pleiotropically affect reproductive isolation" (Servedio et al., 2011). In *Lysimachia arvensis* a more extensive phylogenetic study would be suitable to find out whether the speciation process has finished and to ascertain the role of color polymorphism in that process.

DATA AVAILABILITY STATEMENT

The datasets from this article are publicly available. The microsatellite data in the Zenodo Repository (doi: 10.5281/zenodo.4264724).

AUTHOR CONTRIBUTIONS

MA, PO, and MT planned and designed the research. All authors collected the samples and FJJ-L carried out the inbreeding depression and molecular experiments. FJJ-L, MA, and PO analyzed the data and wrote the first versions of the manuscript that was later edited by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.563110/full#supplementary-material

Supplementary Figure 1 | Proportion of visits per 15-min period at blue-flowered (blue bars) and red-flowered (orange bars) plants of *Lysimachia arvensis* at experimental stands differing in color proportions during two consecutive years (2014 and 2015). Plants were placed in the field and exposed to their natural pollinators. Each stands contained 80 flowers, and plants of different colors were intermingled. Mean and standard errors are shown. Asterisks indicate significant differences between colors in each stands after GLM with binomial distribution of errors and logit link function.

Supplementary Figure 2 | (A) Geographical distribution of the studied populations of *Lysimachia arvensis*. Colors correspond to population type: blue for monomorphic blue, red for monomorphic red, and lilac for polymorphic populations. **(B)** Flowers of the blue and red morphs of *Lysimachia arvensis*.

Supplementary Figure 3 | Genetic diversity accumulation curves for individuals sampled in ten individual populations with different sizes and color-morph frequencies. Genetic diversity is shown for groups, considering population type (monomorphic or polymorphic) and color (blue or red), and for individual populations. Codes of populations appear in **Supplementary Table 2**. The number of individuals at which the number alleles stabilizes represent a sample size large enough to characterize the genetic diversity of the populations.

REFERENCES

Ågren, J., and Schemske, D. W. (1993). Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution* 47, 125–135. doi: 10.1111/j.1558-5646.1993.tb01204.x

Arista, M., Berjano, R., Viruel, J., Ortiz, M. Á, Talavera, M., and Ortiz, P. L. (2017). Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible *Hypochaeris salzmanniana* (Asteraceae). *Ann. Bot.* 120, 447–456. doi: 10.1093/aob/mcx059

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Botes, C., Johnson, S. D., and Cowling, R. M. (2008). Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering phenology. *Oikos* 117, 875–882. doi: 10.1111/j.0030-1299.2008.16391.x

Brito, V. L., Mori, G. M., Vigna, B. B., Azevedo-Silva, M., Souza, A. P., and Sazima, M. (2016). Genetic structure and diversity of populations of polyploid *Tibouchina pulchra* Cogn.(Melastomataceae) under different environmental conditions in extremes of an elevational gradient. *Tree Genetics Genomes* 12, 101. doi: 10.1007/s11295-016-1059-y

Brown, B. A., and Clegg, M. T. (1984). Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea*. *Evolution* 38, 796–803. doi: 10.1111/j.1558-5646.1984.tb00352.x

Bruvo, R., Michiels, N. K., D'Souza, T. G., and Schulenburg, H. (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Mol. Ecol.* 13, 2101–2106. doi: 10.1111/j.1365-294X.2004.02209.x

Brys, R., and Jacquemyn, H. (2016). Severe outbreeding and inbreeding depression maintain mating system differentiation in *Epipactis* (Orchidaceae). *J. Evol. Biol.* 29, 352–359. doi: 10.1111/jeb.12787

Busch, J. W. (2005). Inbreeding depression in self-incompatible and self-compatible populations of Leavenworthia alabamica. *Heredity* 94, 159–165. doi: 10.1038/sj.hdy.6800584

Busch, J. W., and Delph, L. F. (2011). The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. *Ann. Bot.* 109, 553–562. doi: 10.1093/aob/mcr219

Charlesworth, D. (2006). Evolution of plant breeding systems. *Curr. Biol.* 16, R726–R735. doi: 10.1016/j.cub.2006.07.068

Charlesworth, D., and Wright, S. I. (2001). Breeding systems and genome evolution. *Curr. Opin. Genet. Dev.* 11, 685–690. doi: 10.1016/S0959-437X(00)00254-9

Cheptou, P. O., and Donohue, K. (2010). Environment-dependent inbreeding depression: its ecological and evolutionary significance. *New Phytol.* 189, 395–407. doi: 10.1111/j.1469-8137.2010.03541.x

Chesson, P., Gebauer, R. L. E., Schwinning, S., Huntly, N., Wiegand, K., Ernest, M. S. K., et al. (2004). Resource pulses, species interactions, and diversity maintenance in arid and semi-arid environments. *Oecologia* 141, 236–253. doi: 10.1007/s00442-004-1551-1

Clark, L., and Jasieniuk, M. (2011). Polysat: an R package for polyploid microsatellite analysis. *Mol. Ecol. Resour.* 11, 562–566. doi: 10.1111/j.1755-0998.2011.02985.x

Cruz-Neto, O., Machado, I. C., Duarte, J. A., and Lopes, A. V. (2011). Synchronous phenology of hawkmoths (Sphingidae) and Inga species (Fabaceae-Mimosoideae): implications for the restoration of the Atlantic forest of northeastern Brazil. *Biodiv. Conserv.* 20, 751–765. doi: 10.1007/s10531-010-9975-x

Dart, S., and Eckert, C. G. (2013). Experimental and genetic analyses reveal that inbreeding depression declines with increased self-fertilization among populations of a coastal dune plant. *J. Evol. Biol.* 26, 587–599. doi: 10.1111/jeb.12075

Delmas, C. E., Cheptou, P. O., Escaravage, N., and Pornon, A. (2014). High lifetime inbreeding depression counteracts the reproductive assurance benefit of selfing

in a mass-flowering shrub. *BMC Evol. Biol.* 14:243. doi: 10.1186/s12862-014-0243-7

Earl, D. A., and vonHoldt, B. M. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour*. 4, 359–361. doi: 10.1007/s12686-011-9548-7

Ellmer, M., and Andersson, S. (2004). Inbreeding depression in *Nigella degenii* (Ranunculaceae): fitness components compared with morphological and phenological characters. *Int. J. Plant Sci.* 165, 1055–1061. doi: 10.1086/423868

Evanno, G., Regnaut, S., and Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Mol. Ecol.* 14, 2611–2620. doi: 10.1111/j.1365-294X.2005.02553.x

Excoffier, L., Smouseand, P. E., and Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genet* 131, 479–491.

Fady, B., and Conord, C. (2010). Macroecological patterns of species and genetic diversity in vascular plants of the Mediterranean basin. *Divers. Distrib*. 16, 53–64. doi: 10.1111/j.1472-4642.2009.00621.x

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Ann. Rev. Ecol. Syst*. 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Ferguson, L. F. (1972). "*Anagallis* L," in *Flora Europaea*, eds T. G. Tutin, et al. (Cambridge: Cambridge University Press).

Finch-Savage, W. E., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. *New Phytol*. 171, 501–523. doi: 10.1111/j.1469-8137.2006.01787.x

Flint-Garcia, S. A., Thornsberry, J. M., and Buckler, E. S. IV (2003). Structure of linkage disequilibrium in plants. *Annu. Rev. Plant Biol*. 54, 357–374. doi: 10.1146/annurev.arplant.54.031902.134907

Ford, E. B. (1945). Polymorphism. *Biol. Rev*. 20, 73–88. doi: 10.1111/j.1469-185X.1945.tb00315.x

Forsman, A., Ahnesjo, J., Caesar, S., and Karlsson, M. A. (2008). Model of ecological and evolutionary consequences of color polymorphism. *Ecology* 89, 34–40. doi: 10.1890/07-0572.1

Fox, G. A. (2003). Assortative mating and plant phenology: evolutionary and practical consequences. *Evol. Ecol. Res*. 5, 1–18.

Fox, G. A., and Kelly, C. K. (1993). Plant phenology: neutrality and selection. *Trends Ecol. Evol*. 8, 34–35. doi: 10.1016/0169-5347(93)90129-D

Fry, J. D., and Rausher, M. D. (1997). Selection on a floral color polymorphism in the tall morning glory (*Ipomoea purpurea*): transmission success of the alleles through pollen. *Evolution* 51, 66–78. doi: 10.1111/j.1558-5646.1997.tb02389.x

Gigord, L. D., Macnair, M. R., and Smithson, A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the reward less orchid *Dactylorhiza sambucina* (L.) Soo. *Proc. Natl. Acad. Sci. U.S.A*. 98, 6253–6255. doi: 10.1073/pnas.111162598

Giménez-Benavides, L., Escudero, A., and Iriondo, J. M. (2007). Reproductive limits of a late-flowering high-mountain Mediterranean plant along an elevational climate gradient. *New Phytol*. 173, 367–382. doi: 10.1111/j.1469-8137.2006.01932.x

Glémin, S., Bazin, E., and Charlesworth, D. (2006). Impact of mating systems on patterns of sequence polymorphism in flowering plants. *Proc. R. Soc. Lond*. 273, 3011–3019. doi: 10.1098/rspb.2006.3657

Glenner, G. G. (1990). "Formazans and tetrazolium salts," in *Biological Stains*, ed. R. D. Lillie (St. Louis, MO: Sigma Chemical Company), 225–235.

Goslee, S. C., and Urban, D. L. (2007). The ecodist package for dissimilarity- based analysis of ecological data. *J. Stat. Softw*. 22, 1–19. doi: 10.18637/jss.v022.i07

Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F-statistics. *J. Hered*. 86, 485–486. doi: 10.1093/oxfordjournals.jhered.a111627

Hamrick, J. L., and Godt, M. W. (1996). Effects of life history traits on genetic diversity in plant species. *Philos. Trans. R. Soc. B* 351, 1291–1298. doi: 10.1098/rstb.1996.0112

Harborne, J. B. (1968). Comparative biochemistry of the flavonoids—VII: correlations between flavonoid pigmentation and systematics in the family Primulaceae. *Phytochem* 7, 1215–1230. doi: 10.1016/s0031-9422(00)85616-2

Holsinger, K. E. (2000). Reproductive systems and evolution in vascular plants. *Proc. Natl. Acad. Sci. U.S.A*. 97, 7037–7042. doi: 10.1073/pnas.97.13.7037

Husband, B. C., and Schemske, D. W. (1996). Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50, 54–70. doi: 10.2307/2410780

Huxley, J. S. (1955). Morphism and evolution. *Heredity* 9, 1–51. doi: 10.1038/hdy.1955.1

Igic, B., and Busch, J. W. (2013). Is self-fertilization an evolutionary dead end? *New Phytol*. 198, 386–397. doi: 10.1111/nph.12182

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am. Nat*. 165, 225–237. doi: 10.1086/426714

Ishikura, N. (1981). Flavonoids in the petal cells of *Anagallis arvensis* f. *coerulea* containing a blue crystalline anthocyanin. *Z. Pflanzenphysiol.* 103, 469–473. doi: 10.1016/S0044-328X(81)80170-5

Jiménez-López, F. J., Arista, M., Talavera, M., Pannell, J. R., and Ortiz, P. L. (2019a). Heritabilities of lateral and vertical herkogamy in *Lysimachia arvensis*. *Plant Spec. Biol*. 34, 31–37. doi: 10.1111/1442-1984.12229

Jiménez-López, F. J., Matas, L., Arista, M., and Ortiz, P. L. (2020a). Flower colour segregation and flower discrimination under the bee vision model in the polymorphic *Lysimachia arvensis*. *Plant Biosyst.* 154, 535–543. doi: 10.1080/11263504.2019.1651776

Jiménez-López, F. J., Ortiz, P. L., Talavera, M., and Arista, M. (2019b). Selfing maintains flower colour polymorphism in *L. arvensis* despite high inbreeding depression. *bioRxiv* [Preprint]. doi: 10.1101/761122

Jiménez-López, F. J., Ortiz, P. L., Talavera, M., Pannell, J. R., and Arista, M. (2020b). The role of lateral and vertical herkogamy in the divergence of the blue- and red-flowered lineages *of Lysimachia arvensis*. *Ann. Bot*. 125, 1127–1135. doi: 10.1093/aob/mcaa047

Jiménez-López, F. J., Talavera, M., Ortiz, P. L., and Arista, M. (2015). Nuclear microsatellite primers in the annual herb *Lysimachia arvensis* (Myrsinaceae) and closely related taxa. *Biochem. Syst. Ecol*. 58, 242–246. doi: 10.1016/j.bse.2014.12.014

Jones, K. N., and Reithel, J. S. (2001). Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *Am. J. Bot*. 88, 447–454. doi: 10.2307/2657109

Kagawa, K., and Takimoto, G. (2016). Inaccurate color discrimination by pollinators promotes evolution of discrete color polymorphism in food-deceptive flowers. *Am. Nat.* 187, 194–204. doi: 10.1038/s41598-020-66356-4

Kalisz, S., Vogler, D. W., and Hanley, K. M. (2004). Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430:884. doi: 10.1038/nature02776

Kamvar, Z. N., Brooks, J. C., and Grünwald, N. J. (2015). Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Front. Genet*. 6:208. doi: 10.3389/fgene.2015.00208

Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2:e281. doi: 10.7717/peerj.281

Kawecki, T. J., and Ebert, D. (2004). Conceptual issues in local adaptation. *Ecol. Lett*. 7, 1225–1241. doi: 10.1111/j.1461-0248.2004.00684.x

Lande, R., and Schemske, D. W. (1985). The evolution of self-fertilization and inbreeding depression in plants. 1. Genetics models. *Evolution* 39, 24–40. doi: 10.1111/j.1558-5646.1985.tb04077.x

Leimar, O. (2005). The evolution of phenotypic polymorphism: randomized strategies versus evolutionary branching. *Am. Nat*. 165, 669–681. doi: 10.1086/429566

Lowry, D., Modliszewski, J., Wright, K., Wu, C., and Willis, J. (2008). The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philos. Trans. R. Soc. Lond.* 363, 3009–3021. doi: 10.1098/rstb.2008.0064

Manns, U., and Anderberg, A. A. (2009). New combinations and names in *Lysimachia* (Myrsinaceae) for species of *Anagallis*, *Pelletiera* and *Trientalis*. *Willdenowia* 39, 49–55. doi: 10.3372/wi.39.39103

Manzano-Piedras, E., Marcer, A., Alonso-Blanco, C., and Picó, F. X. (2014). Deciphering the adjustment between environment and life history in annuals: lessons from a geographically-explicit approach in *Arabidopsis thaliana*. *PLoS One* 9:e87836. doi: 10.1371/journal.pone.0087836

Martin, N. H., and Willis, J. H. (2007). Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* 61, 68–82. doi: 10.1111/j.1558-5646.2007.00006.x

McLean, C. A., and Stuart-Fox, D. (2014). Geographic variation in animal colour polymorphisms and its role in speciation. *Biol. Rev*. 89, 860–873. doi: 10.1111/brv.12083

Medel, R., Botto-Mahan, C., and Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* 84, 1721–1732. doi: 10.1890/01-0688

Meirmans, P. G., and Van Tienderen, P. H. (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Mol. Ecol. Notes* 4, 792–794. doi: 10.1111/j.1471-8286.2004.00770.x

Meléndez-Ackerman, E., Campbell, D. R., and Waser, N. M. (1997). Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* 78, 2532–2541. doi: 10.2307/2265912

Monein, A., Atta, I. I., and Shehata, A. A. (2003). On the delimitation of *Anagallis arvensis* L. (Primulaceae). Evidence based on micromorphological characters, Palynological features and Karyological studies. *Pak. J. Biol. Agric. Sci*. 6, 29–35. doi: 10.3923/pjbs.2003.29.35

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol*. 20, 8–20. doi: 10.1111/plb.12575

Nosil, P., Harmon, L. J., and Seehausen, O. (2009). Ecological explanations for (incomplete) speciation. *Trends Ecol. Evol.* 24, 145–156. doi: 10.1016/j.tree.2008.10.011

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2013). *Package 'Vegan': Community Ecology Package, Version 2*. 1–295. doi: 10.1016/j.tree.2008.10.011

Ollerton, J., and Lack, A. (1998). Relationships between flowering phenology, plant size and reproductive success in *Lotus corniculatus* (Fabaceae). *Plant Ecol*. 139, 35–47. doi: 10.1023/A:1009798320049

Ortiz, P. L., Berjano, R., Talavera, M., Rodríguez-Zayas, L., and Arista, M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: how is the red morph maintained in Mediterranean environments? *Perspect. Plant Ecol. Evol. Syst*. 17, 142–150. doi: 10.1016/j.ppees.2015.01.004

Pritchard, J. K. (2010). *Documentation for STRUCTURE Software: Version 2.3*. Available online at: https://web.stanford.edu/group/pritchardlab/structure_software/release_versions/v2.3.4/html/structure.html

Pujadas, A. (1997). "*Anagallis* L," in *Flora Iberica*, Vol. V, eds S. Castroviejo, et al. (Madrid: Real Jardín Botánico, CSIC).

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci*. 169, 7–21. doi: 10.1086/523358

Rausher, M. D., Augustine, D., and VanderKooi, A. (1993). Absence of pollen discounting in a genotype of *Ipomoea purpurea* exhibiting increased selfing. *Evolution* 47, 1688–1695. doi: 10.1111/j.1558-5646.1993.tb01261.x

Rodríguez-Pérez, J., and Traveset, A. (2016). Effects of flowering phenology and synchrony on the reproductive success of a long-flowering shrub. *AoB Plants* 8:lw007. doi: 10.1093/aobpla/plw007

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* 55, 1269–1282. doi: 10.1111/j.0014-3820.2001.tb00650.x

Schenk, J. H., and Jackson, R. B. (2002). The global biogeography of roots. *Ecol. Monogr.* 72, 311–328. doi: 10.1890/0012-9615(2002)072[0311:tgbor]2.0.co;2

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol*. 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Schneider, S., Roessli, D., and Excoffier, L. (2000). *Arlequin: a Software for Population Genetics Data Analysis. User Manual v. 2000*. Switzerland: University of Geneva.

Schoen, D. J., and Brown, A. H. D. (1991). Whole- and part-flower self-pollination in *Glycine clandestina* and *G. argyrea* and the evolution of autogamy. *Evolution* 45, 1665–1674. doi: 10.1111/j.1558-5646.1991.tb02670.x

Servedio, M. R., Van Doorn, G. S., Kopp, M., Frame, A. M., and Nosil, P. (2011). Magic traits in speciation: 'magic'but not rare? *Trends Ecol. Evol*. 26, 389–397. doi: 10.1016/j.tree.2011.04.005

Shivanna, K. R. (2015). Reproductive assurance through autogamous self-pollination across diverse sexual and breeding systems. *Curr. Sci*. 109, 1255–1263. doi: 10.18520/v109/i7/1255-1263

Sletvold, N., Grindeland, J. M., Zu, P., and Ågren, J. (2012). Strong inbreeding depression and local outbreeding depression in the rewarding orchid *Gymnadenia conopsea*. *Conserv. Genet.* 13, 1305–1315. doi: 10.1007/s10592-012-0373-7

Sobral, M., Veiga, T., Dominguez, P., Guitián, J. A., Guitián, P., and Guitián, J. (2015). Selective pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS One* 10:e0132522. doi: 10.1371/journal.pone.0132522

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 120–135.

Subramaniam, B., and Rausher, M. D. (2000). Balancing selection on a floral polymorphism. *Evolution* 54, 691–695. doi: 10.1111/j.0014-3820.2000.tb00070.x

Takebayashi, N., and Morrell, P. P. (2001). Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *Am. J. Bot*. 88, 1143–1150. doi: 10.2307/3558325

Talavera, M., Jiménez-López, F. J., Ortiz, P. L., and Arista, M. (2020). *Phylogeny and Taxonomic Implications of the Mediterranean Anagallis (Currently in Lysimachia) Sesbe VII*. Seville: Spanish Society of Evolutionary Biology, 135.

Vaidya, P., McDurmon, A., Mattoon, E., Keefe, M., Carley, L., Lee, C. R., et al. (2018). Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (*Boechera stricta*). *New Phytol.* 218, 380–392. doi: 10.1111/nph.14998

Van Puyvelde, K., Van Geert, A., and Triest, L. (2010). ATETRA, a new software program to analyse tetraploid microsatellite data: comparison with TETRA and TETRASAT. *Mol. Ecol. Res*. 10, 331–334. doi: 10.1111/j.1755-0998.2009.02748.x

Voillemot, M., and Pannell, J. R. (2017). Inbreeding depression is high in a self-incompatible perennial herb population but absent in a self-compatible population showing mixed mating. *Ecol. Evol.* 7, 8535–8544. doi: 10.1002/ece3.3354

Warnes, G., and Leisch, F. (2005). Genetics: population genetics. *R Package Version* 1, 1–5.

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol*. 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Waser, N. M., and Price, M. V. (1983). Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302:422. doi: 10.1038/302422a0

Whibley, A. C., Langlade, N. B., Andalo, C., Hanna, A. I., Bangham, A., Thébaud, C., et al. (2006). Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* 313, 963–966. doi: 10.1126/science.1129161

Whitney, K. D. (2005). Linking frugivores to the dynamics of a fruit color polymorphism. *Am. J. Bot*. 92, 859–867. doi: 10.3732/ajb.92.5.859

Widmer, A., Lexer, C., and Cozzolino, S. (2009). Evolution of reproductive isolation in plants. *Heredity* 102, 31–38. doi: 10.1038/hdy.2008.69

Winn, A. A., Ell, E., Kalisz, S., Cheptou, P. O., Eckert, C. G., Goodwillie, C., et al. (2011). Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* 65, 3339–3359. doi: 10.1111/j.1558-5646.2011.01462.x

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Flower Diversification Across "Pollinator Climates": Sensory Aspects of Corolla Color Evolution in the Florally Diverse South American Genus *Jaborosa* (Solanaceae)

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Flower phenotype may diverge within plant lineages when moving across "pollinator climates" (geographic differences in pollinator abundance or preference). Here we explored the potential importance of pollinators as drivers of floral color diversification in the nightshade genus *Jaborosa,* taking into account color perception capabilities of the actual pollinators (nocturnal hawkmoths vs. saprophilous flies) under a geographic perspective. We analyzed the association between transitions across environments and perceptual color axes using comparative methods. Our results revealed two major evolutionary themes in *Jaborosa*: (1) a "warm subtropical sphingophilous clade" composed of three hawkmoth-pollinated species found in humid lowland habitats, with large white flowers that clustered together in the visual space of a model hawkmoth (*Manduca sexta*) and a "cool-temperate brood-deceptive clade" composed of largely fly-pollinated species with small dark flowers found at high altitudes (Andes) or latitudes (Patagonian Steppe), that clustered together in the visual space of a model blowfly (*Lucilia* sp.) and a syrphid fly (*Eristalis tenax*). Our findings suggest that the ability of plants to colonize newly formed environments during Andean orogeny and the ecological changes that followed were concomitant with transitions in flower color as perceived by different pollinator functional groups. Our findings suggest that habitat and pollination mode are inextricably linked in the history of this South American plant lineage.

Keywords: floral evolution, floral reflectance, pollinator vision, pollinators' color space, hawkmoth pollination, saprophilous fly pollination, pollinator climates

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INTRODUCTION

In a seminal study on pollinator-driven flower diversification, Grant and Grant (1965) coined the expression "pollinator climate" to explain potential selective forces driving floral specialization on different kinds of pollinators (or to self-pollination) across their geographical ranges. Concerning selective pressures, Grant and Grant (1965) stated that latitudinal or altitudinal differences in

pollinator abundance-pollinator climate-are to floral phenotype as environmental factors are to vegetative phenotype. Consequently, as any given abiotic environment may have several covarying dimensions such as temperature, wind and humidity that define a multidimensional selective context, pollinators likewise impose, through their own integrated phenotypes of anatomical and behavioral peculiarities, a multidimensional biotic selective context. More recently, the factors contributing to pollinator climate have been formalized as dimensions of pollinator niche space (Johnson, 2010; Phillips et al., 2020). The pollinator climate/niche approach has been used to formulate and test predictions for local pollinator specialization among populations of specific plants (e.g., *Erica plukenetii* in the western Cape region of South Africa; Van Der Niet et al., 2014) as well as pollination themes in plant communities from different regions (e.g., short vs. long-tongued pollinator guilds; Johnson et al., 2017). One factor that unites the emerging concept structure of pollinator climate/niche with the formalization of pollinator "functional groups" (Fenster et al., 2004) is the expectation that the sensory biases and capabilities of pollinators constitute fundamental dimensions of pollinator climate/niche, leading to consistent behavioral preferences for certain kinds of flowers, the "attraction" component of Grant's body of work on floral isolation (Grant, 1994; Hodges et al., 2004).

An important aspect in considering the role of pollinators as drivers of floral diversification is the context in which diversification is promoted. When considering the geographic perspective, it is possible to distinguish whether floral divergence is or is not associated with changes in the local pollinator context (see Ellis and Johnson, 2009). For example, floral diversification may occur when plants disperse to geographical areas where the pollinator fauna is depauperate in relation to the source area (Streisfeld and Kohn, 2007). Southern South America is one of the few places in the world where subtropical lowlands lie in close geographical proximity to glaciated high mountain ranges, providing the rare opportunity to assess the influence of markedly contrasting eco-geographic domains in floral diversification mediated by pollinator climates. The topographical and environmental changes that occurred during the Andean orogeny have been crucial both in the origin of new biomes and in the concurrent diversification of Andean lineages (Chaves et al., 2011; Luebert and Weigend, 2014; Strelin et al., 2017). Studies on the paleobotany and geology of South America suggest that up to half of the Central Andes uplift (i.e., a rise from 2000 m to the present 4000 m) has taken place in the last 10 Ma. The combination of maximum altitudes and globally cooling temperatures date the origins of alpine Andean vegetation to ca. 3–5 Ma, making it the youngest natural ecosystem of South America (Graham, 2009). The formation of alpine and steppe habitats with cold desert climate about 3–5 Ma bp challenged resident organisms with a completely new context to which to adapt. For plants, the pollination environment changed dramatically. The emergence of these new habitats represented an unprecedented adaptive challenge with regard to pollination climate. Flies, comprising between 67 and 77% of the pollinator fauna diversity, are the dominant pollen vectors both in alpine (see Arroyo et al., 1982; Devoto et al., 2005) and high latitude environments (Elberling and Olesen, 1999). In contrast, other animals are dominant pollinators in the sub-alpine shrub zone and the subtropical lowland areas where bees (Devoto et al., 2005; Chacoff et al., 2012) or nocturnal hawkmoths (Sazatornil et al., 2016), respectively, are relatively more important pollinators. Consequently, we would expect that plants evolving in an area that has markedly changed in pollinator climate would be under selection to adjust their flower phenotypes accordingly.

Floral color is an important visual signal for pollinator attraction. Because different animal lineages possess distinctive color-sensitive visual receptors and cognitive capabilities, the way each pollinator group (e.g., bats, hummingbirds, flies, lepidopterans, bees) perceives color is unique (Briscoe and Chittka, 2001; Schaefer and Ruxton, 2011). Considering the hypothesis that flower color signals evolved to match the visual systems of pollinators (Chittka and Menzel, 1992), it is expected that flowers present corolla colors that exploit their pollinators' visual capability and preferences (De Ibarra and Vorobyev, 2009; Shrestha et al., 2013; Paine et al., 2019). However, pollinator attraction is not the only mechanism driving the evolution of flower color. Several case studies have shown that corolla color diversification can result from pollinator shifts (=attracting different functional groups; review by Rausher, 2008), from interspecific competition among pollinators of the same functional group (=promoting floral constancy; Muchhala et al., 2014) or from avoiding antagonistic flower visitors (=reducing florivory or larceny; Irwin et al., 2003; Gutierrez De Camargo et al., 2019; Chen et al., 2020).

Floral traits in most plant species have the potential to adapt to new conditions under changing selective pollination environments (Kay et al., 2005; Whittall and Hodges, 2007). Such shifts in pollination systems may occur quite rapidly through novel floral mutations. For example, a single allelic substitution is sufficient to explain flower color change in monkey flowers, representing an adaptive shift between bumblebees and hummingbirds as pollinators (Bradshaw and Shemske, 2003). Flower color is a labile trait and several studies have shown that color change is spurred by adaptive pollinator shifts, i.e., a given color evolved in concert with pollination by a specific functional group of pollinators. Some of the most common transitions of pollination systems are from bee to moth, bee to bird and bird to moth (review by Rosas-Guerrero et al., 2014). However, only one case of transition between brood site deception by saprophilous fly and nocturnal hawkmoth -and vice-versa- has been reported so far in a complex of milkweed species in Japan (Yamashiro et al., 2008). Flowers that use brood-site deception to attract saprophilous flies as pollinators are well known for their unusual floral morphology, visual and olfactory display (review by Urru et al., 2011). These two modes of pollination are so utterly different (brood site deception of saprophilous flies vs. honest –nectar rewarding– pollination by hawkmoths) as to require, in theory, many changes in floral phenotype to integrate the sensory cues that attract pollinators with the functional morphology necessary to effect pollen transfer (Phillips et al., 2020). In this study, we explore a putative moth-fly pollinator shift from the standpoint of flower color, in the South American genus *Jaborosa*.

The genus *Jaborosa* Juss. (Solanaceae) is endemic to southern South America and exhibits strong inter-specific variation in floral traits across geographic domains. Such variation ranges from night-blooming white fragrant flowers with very long corolla tubes (Vesprini and Galetto, 2000) that produce abundant nectar, to diurnal, dark pigmented, unpleasant smelling flowers with shallow corollas that produce little to no nectar (Moré et al., 2013, 2019). Two utterly different pollination modes have been described associated with these extreme phenotypes: nectar-rewarding flowers pollinated by nocturnal hawkmoths and rewardless brood-site deceptive flowers pollinated by saprophilous flies, respectively. Geographical distribution of hawkmoth-pollinated species is restricted to humid subtropical lowlands, whereas carrion-fly pollinated species grow in semiarid regions located either at high altitudes in the Andes or at high latitudes in the Monte desert and the temperate Patagonian steppe of southernmost Argentina (Moré et al., 2015). Given the floral diversity and present geographic distribution of the genus *Jaborosa* as well as the geological history of the region now occupied by *Jaborosa*, we expect that flower evolution in *Jaborosa* reflects ancestral movement across contrasting pollinator climates. In particular, floral evolution should have been subjected to shifts along the perceptual dimension of the changing pollinator climates. Changes in flower signaling should bear on visual conspicuousness in terms of chromatic and achromatic contrast vis-à-vis the perceptual capabilities of the geographically and historically changing pollinators. Consequently, we first expect color conspicuousness to be greater in plant species with nocturnal flowers than in those with diurnal ones (see Kuenzinger et al., 2019). Second, we expect the timing of the shifts to be consistent with the geological events that created new pollination climates. Third, we expect that the phenotypic transitions across pollination climates occurred along perceptual dimensions of the changing pollinators. In the present study we use plant-phylogenetically informed and pollinator-visual modeling approaches to assess the evolution of color diversity within the genus *Jaborosa*, taking into account the color perception capabilities of the current pollinators serving as potential selective agents.

MATERIALS AND METHODS

Study System

The nightshade genus *Jaborosa* comprises 22 species that exhibit remarkable interspecific variation in floral traits (**Figure 1**). Previous phylogenetic analyses recovered two strongly supported clades with geographic affinities: a "subtropical lowland clade," including the three species with sphingophilous (hawkmoth-pollinated) flowers inhabiting lowland areas, and an "andean clade," including the remaining species, mainly distributed across the higher altitudes (Andes mountain range) and higher latitudes (temperate South America) (Moré et al., 2015). A phylogenetic hypothesis for *Jaborosa* was estimated using Bayesian inference from the four-gene plastid dataset collected from 30 taxa, including 19 *Jaborosa* species and 11 representatives of outgroup genera (Moré et al., 2015). The Bayesian analysis was performed using BEAST v.1.7.5 (Drummond et al., 2012) under a relaxed clock model with branch-specific rates following a lognormal distribution and a GTR+G model of nucleotide substitution with a randomly generated starting-tree topology and Yule speciation process. The topology shown here is a maximum clade credibility (MCC) tree pruned to show only the 11 taxa studied here using the phytools 0.7-20 package (Revell, 2012) of R software (R Core Team, 2020; **Figure 1**).

Pollinators

Field studies were conducted in several localities of Argentina to characterize the pollinators of each species of *Jaborosa*. Mean observation time per species was 223 min, ranging from 60 to 630 min (**Supplementary Table 1**). Floral visitors were recorded during day and nighttime (whenever climatic conditions allowed) by direct observation, photography and video in the field. Representative specimens of insects visiting the flowers were captured using a hand-held net for later identification, with voucher specimens deposited at the Laboratorio de Ecología Evolutiva y Biología Floral (IMBIV, Córdoba, Argentina). Additionally, stigmas were inspected for the presence of lepidopteran scales in the field using a hand lens (20×), as evidence of recent visitation. Nocturnal sampling of hawkmoth individuals was carried out by means of light traps in two localities (Termas de Reyes and El Fuerte populations, Jujuy Province; see Moré et al., 2006 for details) within the range of *Jaborosa integrifolia*. Hawkmoths were collected for identification of pollen loads in the laboratory as described by Schlumpberger et al. (2009).

Spectra Acquisition and Processing

We measured corolla reflectance across 11 *Jaborosa* species representing the full spectrum of floral coloration in the genus (**Figure 1** and **Supplementary Table 1**). Measurements were taken from fresh flowers (4–11 individuals per species and 1–3 flowers per individual when available) and leaves (1–3 per species) as background against which flowers are displayed. We used SpectraSuite software (Ocean Insights, Rostock, Germany) for data acquisition with a boxcar width of 100 nm and an integration time of 2 s per scan. The percentage of light reflected was measured every 0.20 nm using an USB4000 miniature fiber optic spectrophotometer with a deuterium-tungsten halogen lamp to provide standardized illumination and a UV-visible reflection/backscatter probe with a wavelength range between 300 and 1100 nm (Ocean Insights, Rostock, Germany). The reflected light was collected at 45° to avoid specular reflectance. The probe was mounted on a prismatic holder and the distance to the corolla surface was 1 mm. A white standard (WS-1-SS White Standard, Ocean Optics Inc., Dunedin, FL, United States) was used to re-calibrate the equipment between each measurement in order to correct for possible shifts in spectrophotometer performance (Chittka and Kevan, 2005). Processing and visualization of the reflectance spectra were done using the pavo 2.2.0 package (Maia et al., 2019) of R software (R Core Team, 2020). Spectra were trimmed to the insect vision range, that includes human visible light and UV light (300–700 nm) using as.rspec function. Spectra were smoothed using local regression as implemented in the procspec function with span set to 0.25 because it was the minimum amount

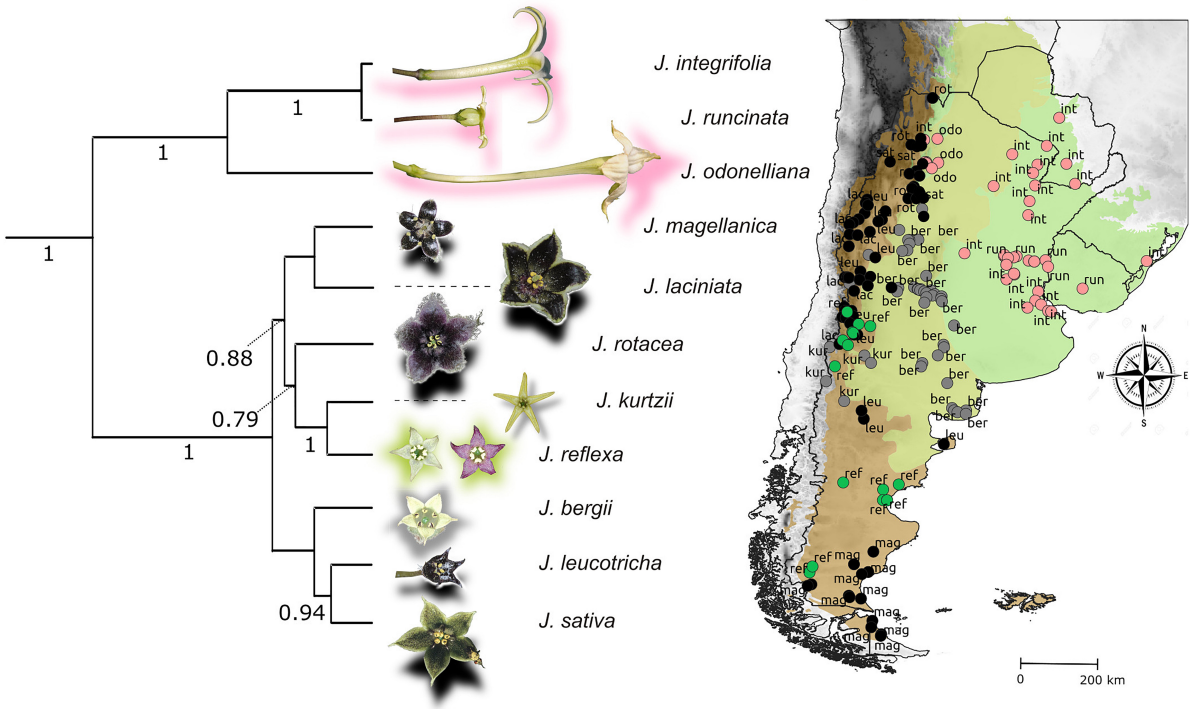


FIGURE 1 | Study system. Flower coloration, phylogenetic relationships and geographical distribution of *Jaborosa* species. Color shading in the map shows the ecoregions classification proposed by Olson et al. (2001) as follows: herb green: lowlands; olive green: subalpine; brown: alpine (see **Supplementary Table 2** for details). Colors of the circles show pollination mode: pink for nocturnal hawkmoths, black for saprophilous flies, green for generalized pollination by small insects, and gray for unknown pollinators, respectively. Numbers in the tree show branch support; only Bayesian posterior probabilities higher than 0.75 are shown.

to remove spectral noise while preserving the original spectral shape. Measurements were averaged by individual and species, or by color type, individual and species for *Jaborosa laciniata* and *Jaborosa reflexa*, respectively, using the aggspec function.

Corolla Reflectance Variation Among *Jaborosa* Species

We examined overall differences in reflectance spectra among the 11 *Jaborosa* species using Principal Component Analysis (PCA), which allowed us to summarize information of complex spectra in a few orthogonal variables while making no assumptions about the receiver's visual system. We analyzed a total of 114 floral spectra that were centered to have a mean reflectance of zero and then binned into 10 intervals of 40-nm to obtain a dataframe suitable for PCA. Performing the PCA with standardized spectral data removes total reflectance as a dominant variable and allows comparing only the spectral shape (Cuthill et al., 1999; Maia et al., 2019).

Evolution of Corolla Reflectance and Pollination Mode Among *Jaborosa* Species

We followed two different approaches to explore the evolution of corolla spectral shape among *Jaborosa* species. First, we represented overall variation in corolla color among species in a phylomorphospace (Chartier et al., 2014). Thus, a time-calibrated phylogeny enabled us to highlight trends in corolla color diversification in relation to species and pollination mode in the biplot resulting from the PCA analyses. The phylomorphospace was constructed using the mean loading values of the first two principal components (PCs) of each species and the MCC tree from the four-gene plastid dataset.

In a second step we estimated the ancestral reconstruction for corolla color and pollination mode. The best model of character evolution for the spectral shape (i.e., PC1 scores from the standardized PCA analysis) was determined using the *fitContinuous* function as implemented in "geiger" v.2.0.6 (Harmon et al., 2008). Three different models were fitted: Brownian motion (BM; Felsenstein, 1973), Ornstein–Uhlenbeck (OU; Hansen, 1997), and early burst (EB; Harmon et al., 2010). The BM model was chosen because, although it showed the same value as OU (σ^2=40.93) it was identified as having the lowest AICc value. An estimation of the ancestral spectral shape was obtained using *anc.ML* function as implemented in "phytools" v.0.7-20 package (Revell, 2012). Model fitting of the pollination mode (discrete trait) was done using the *ace* function as implemented in "ape" v.5.3 (Paradis et al., 2004) under three different models: equal rates, symmetrical rates and all rates different. No significant differences were found after performing an ANOVA among the likelihood values of the different models. Thus, the equal rates model was chosen because it had the least number of estimated parameters. Character-state history among pollination modes was traced using a Bayesian stochastic character mapping approach using the *make.simmap* function

from "phytools." Multiple estimates were performed (N = 100) in order to provide the probability and confidence intervals of ancestral states at nodes (Bollback, 2006).

Visual Modeling

Given that different animals process and perceive flower colors differently, we represented the corolla reflectance spectra in the visual perceptual spaces of the two main pollinator groups (nocturnal hawkmoths and saprophilous flies) and one occasional pollinator (the syrphid *Eristalis tenax*), covering the spectrum of pollinator functional groups recorded for the 11 *Jaborosa* species studied here(see section "Results"). This procedure allowed us to examine both how intra- and interspecific corolla color variation within the genus is perceived by these different functional groups (review by Renoult et al., 2017) and how such variation changed within the perceptual space of different pollinator functional groups during the diversification of the genus *Jaborosa*. We used two different kinds of models of color vision: the categorical perceptual spaces modeled for blowflies (*Lucilia* sp., Calliphoridae; Troje, 1993) and for hoverflies (*E. tenax*, Syrphidae; Lunau, 2014; Shrestha et al., 2016), respectively, and the Maxwell color triangle of a nocturnal hawkmoth (*Manduca sexta*, Sphingidae, Lepidoptera). We estimated quantum catches of each photoreceptor for the two model classes (fly and hawkmoth) incorporating a von Kries transformation that normalizes receptor quantum catches to the background (Maia et al., 2019). We used as background the average spectrum of the leaves from all *Jaborosa* species. According to the model proposed by Troje (1993), blowflies exhibit a categorical color vision system based on the relative excitations of the two pale-type (p+, p−) and two yellow type (y+, y−) receptors. The receptor of each pair that is stimulated more strongly determines which color the fly perceives. Thus, flies perceive four color categories: fly-UV (p+, y+), fly-blue (p−, y+), fly-yellow (p−, y−), and fly-purple (p+, y−). To plot corolla spectra from the *Jaborosa* species as loci in the fly vision models (blowfly and hoverfly), the quantum catches of each of the four photoreceptors were calculated by integrating the product of photoreceptor sensitivities, the standard D65 illumination and the corolla spectral reflectance. The photoreceptor sensitivities used for the blowfly *Lucilia* sp. follow Hardie and Kirschfeld (1983) and those for the hoverfly *E. tenax* follow Lunau (2014) and Shrestha et al. (2016). Stimuli with loci falling within the same color category are assumed to be indistinguishable to the fly (Troje, 1993; Arnold et al., 2009).

In contrast, hawkmoths have superposition eyes with three different photoreceptor types most sensitive in the ultraviolet (UV), blue (B), and green (G) ranges of the spectrum (White et al., 1994). We calculated the photoreceptor responses of *M. sexta* (UV = 357 nm, Blue = 450 nm, and Green = 520 nm; Bennett and Brown, 1985) and flower color loci were placed within the Maxwell color triangle. Each corner of the triangle corresponds to one of the three photoreceptor types of the moth eye and represents a pure color that only excites this receptor type. The location of a color locus in the triangle represents the relative excitation of the three receptor types by that floral sample (Balkenius et al., 2004). The light spectrum typical for sunset was used as ambient illumination (Henze et al., 2018), because hawkmoth foraging begins from sunset to dusk in this biome (Moré et al., 2006), although it can continue through the night (Vesprini and Galetto, 2000).

We estimated floral color conspicuousness for *M. sexta* under the Receptor Noise Limited (RNL) model of Vorobyev and Osorio (1998), in terms of chromatic and achromatic contrasts against the green leaves as background. This model has been behaviorally validated under different color stimulus choices for several species of diurnal and nocturnal hawkmoths (e.g., Kelber et al., 2002; Johnsen et al., 2006) including *M. sexta* (Kuenzinger et al., 2019). However, discrimination ability in the long wavelength range depends on both achromatic and chromatic differences (Telles et al., 2014, 2016). Chromatic contrast describes the color contrast that excludes luminance information while the achromatic contrast refers to the luminance difference between a flower color and its background. For chromatic contrast we used the following parameters: Weber fraction of 0.1 as empirically estimated for the tiger moth *Arctia plantaginis* (Henze et al., 2018); noise was set as "neural"; photoreceptor densities UV = 0.1, B = 0.23, L = 0.67 based on data from the ventral portion of the compound eye of *M. sexta* (White et al., 2003); and quantum catch was set to "Qi." Achromatic contrast was calculated as the contrast produced in the long-wave photoreceptor (Henze et al., 2018). All calculations were performed using the pavo v.2.2.0 package (Maia et al., 2019) of R software (R Core Team, 2020). In order to determine whether samples of the *Jaborosa* species and pollination mode are discriminable in the hawkmoth's color space, all pairwise chromatic distances measured in Just Noticeable Differences (JND) among species samples and pollination modes were calculated. A multi-response permutation procedure was then applied using a PERMANOVA with *bootcoldist* function where the observed pairwise mean color distance was compared with a distribution obtained by randomly assigning observed colors among samples. One thousand pseudo-values were obtained in this way, and the observed value was considered significant if it was greater than 95% of the pseudo-values. We considered this analysis to be adequate because it takes into account the multivariate nature of the data (Maia and White, 2018).

Finally, we estimated floral conspicuousness for the fly by calculating Euclidean distances among color loci as recently proposed by Hannah et al. (2019). Hannah et al. (2019) showed that color choices among different blue and yellow stimuli by the hoverfly *E. tenax* are mediated by a continuous monotonic function. These authors proposed that the Troje (1993) model of fly color processing could be a useful template for mapping how fly pollinators might discriminate flower colors from a background stimulus falling within the same quadrant.

Evolution of Color Phenotype Across Environments

To test whether, along with evolutionary diversification in *Jaborosa*, the ability to colonize newly formed environments was concomitant with adjustment in flower color as is perceived by pollinators, we analyzed the association between transitions

across environments and perceptual color axes. As formation of the new environments was synchronous with the uplifting of the Central Andes and rain shadowing of its eastern slopes during the last 10 Ma (see section "Discussion"), the initial humid lowland forests and grasslands were succeeded by dry mid-altitude scrub and subsequently by cold alpine and high latitude cold deserts (Salzmann et al., 2008; Strelin et al., 2017). For the purpose of analysis, we classified the area now occupied by *Jaborosa* in three main environmental zones that are consistent with groups of widely recognized ecoregions in South America (see Olson et al., 2001 and **Supplementary Table 2**): (1) humid lowland zone, (2) dry lowland and foothill zone, and (3) alpine and high latitude zone. These zones range in pollinator environment from hawkmoth-dominated to fly-dominated pollinator climates (see Arroyo et al., 1982; Elberling and Olesen, 1999; Devoto et al., 2005; Chacoff et al., 2012; Sazatornil et al., 2016; Moré et al., 2019). This classification is constrained by the relevant groups of pollinators for *Jaborosa* species and does not include Hymenoptera and vertebrates (bats, hummingbirds, and rodents) that are also important pollinators in the dry lowland and high altitude habitats of southern South America, respectively (Strelin et al., 2017; Dellinger et al., 2019).

The ability of extant *Jaborosa* species and their reconstructed ancestors to occupy different environmental zones was explored in a threshold model which uses Bayesian Markov chain Monte Carlo (MCMC) to sample the liabilities from joint posterior probability distributions (Wright, 1934; Revell, 2014). The threshold model is inherently ordered, thus the three environmental zones were ordered according to their chronological appearance as (1) < (2) < (3). To test whether evolutionary changes in environmental occupancy were associated with orderly transitions along pollinator's perceptual axes, we carried out phylogenetic generalized least squares (PGLS) correlations between liabilities to occupy environments and perceptual color vectors. We constructed color vectors using as variables the relativized photon catches (Qi) for hawkmoth and the Euclidean distances for flies, which were obtained from the above visual models. We undertook a Principal Component Analyses (PCA) of Qi values (hawkmoth) and Euclidean distances (blowfly) and the scores of the PC1 axis were used as the color vector. Two evolutionary models were fitted for correlated evolution: BM (Felsenstein, 1973) and OU (Hansen, 1997). The OU model was chosen because it was identified by AIC as the best fit. Analyses were carried out with functions *ancThresh* and *phyl.vcv* in phytools 0.7-20 package (Revell, 2012) of R software (R Core Team, 2020).

RESULTS

Pollinators

The *Jaborosa* species studied were pollinated by insects that could be assigned to three functional groups: saprophilous flies (F), nocturnal hawkmoths (H), and generalized pollination (G). We recorded mostly saprophilous flies as pollinators of five *Jaborosa* species: *J. laciniata*, *Jaborosa leucotricha*, *Jaborosa magellanica*, *Jaborosa rotacea* and *Jaborosa sativa*. Flies belonged to three superfamilies: Muscoidea (Anthomyiidae and Muscidae), Oestroidea (Calliphoridae, Sarcophagidae, and Tachinidae) and Tabanoidea (Tabanidae) and consistently acquired pollen on the dorsal surfaces of their thoraces (nototribic deposition). *J. integrifolia* was pollinated at night by *Manduca tucumana* (Lepidoptera: Sphingidae). A total of 323 nocturnal hawkmoths belonging to 14 species were captured at two western populations of *J. integrifolia*; El Fuerte and Termas de Reyes. Of this sample, seven individuals of the most abundant hawkmoth species, *M. tucumana* (N = 125, mean proboscis length = 79.42 mm), carried pollen loads of *J. integrifolia* on their probosces. We did not record any hawkmoth visits nor the presence of moth scales on floral stigmas in eastern populations (Diamante and Victoria) of *J. integrifolia* during two different years. However, a previous study conducted near our field sites documented rare but effective hawkmoth pollination of *J. integrifolia* (Vesprini and Galetto, 2000). During daytime, a melyrid beetle (*Astylus quadrilineatus*) was commonly observed visiting flowers of both *J. integrifolia* and *Jaborosa runcinata* in two populations (Diamante and Victoria) where these species grow in sympatry. Beetles were observed in high numbers within the flowers (up to 10 individuals were recorded in a single flower of *J. integrifolia*) either feeding on pollen or copulating. The presence of moth scales on the flower stigmas of *Jaborosa odonelliana* and *J. runcinata* provides indirect evidence of moth visitation at night. *J. reflexa* demonstrated generalized pollination by small insects, including syrphid and sarcophagid flies and halictid bees. Ants were observed patrolling the leaves and flowers of two species (*Jaborosa bergii* and *J. rotacea*) but did not contact the anthers and stigmatic surfaces. Finally, we did not observe any floral visitors for two of the studied species, *J. bergii* and *J. odonelliana*, neither did we observe any visitors for the green (polymorphic) *J. laciniata* flowers nor the maroon flowers of *J. reflexa* (**Supplementary Table 1**).

Corolla Reflectance Variation

We recorded variation in corolla reflectance spectra both among *Jaborosa* species and among individuals within species (**Figure 2**). Species pollinated or putatively pollinated by nocturnal hawkmoths (*J. integrifolia*, *J. odonelliana*, and *J. runcinata*) showed corollas with maximum reflectance (50–80% of white standard) between 450 and 700 nm, absorbing in the UV-region of the spectrum, between 300 and 399 nm, and perceived as white by human vision. Diurnal species showed corollas with very low overall reflectance and maximum reflectance of approximately 20% of the white standard. Saprophilous fly-pollinated species were maroon to black in color as perceived by human vision (*J. laciniata*, *J. leucotricha*, *J. rotacea*, *J. magellanica*) or pale green freckled with dark blotches (*J. sativa*). Two species showed intraspecific variation in corolla color. *J. laciniata* was polymorphic, with flower colors varying among individuals between black or more rarely green. *J. reflexa* was heteromorphic in flower coloration, with individuals showing flowers of the same age either yellowish to pale green or maroon (**Figures 1**, **2**). Thus, in these two species, we considered flower types separately for subsequent analyses.

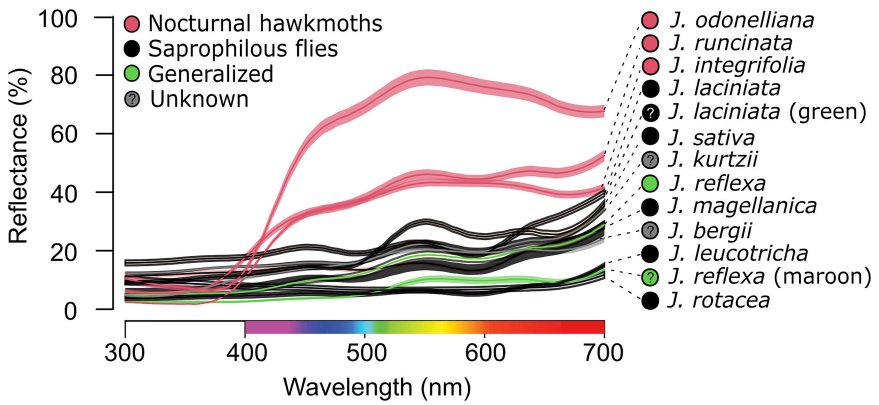


FIGURE 2 | Averaged corolla reflectance of *Jaborosa* species. Lines and shadowed areas show average spectra and standard deviation per species. Colors show pollination mode: pink for hawkmoths, black for saprophilous flies, green for generalized, and gray for unknown pollinators, respectively. Question marks in *J. laciniata* green morph and *J. reflexa* maroon morph stand for unknown pollinators.

The first two PCs of the PCA accounted for 95.61% of total variation in spectral shape among *Jaborosa* species and among individuals within each species. Variation in flower reflectance was detected in PC1 according to pollination mode, loadings of the three species with nocturnal hawkmoth-pollinated flowers clustered together and separated from the plant species with diurnal flowers (five saprophilous fly-pollinated species, the one with generalized pollination and the two species with unknown pollinators; **Figure 3A**). The phylomorphospace showed that variation in reflectance spectra is associated with clades, with the three species comprising the humid lowland clade showing high values of PC1 loading scores and the Andean clade with low values of PC1 loading scores (**Figure 3B**).

Evolution of Corolla Reflectance and Pollination Mode Among *Jaborosa* Species

Our results show that diversification within the Andean clade is associated with a pollinator shift from nocturnal hawkmoths to saprophilous flies and occurred in concert with changes in corolla coloration – from white flowers to either maroon and black flowers or pale green freckled with dark blotches – during the initial diversification of *Jaborosa* ancestors across southern South America (**Figure 3B**).

The most recent common ancestor (MRCA) of extant *Jaborosa* was reconstructed as bearing corollas with low reflectance in the medium- and long-wavelength regions and absorbing in the UV and short-regions (perceived as whitish to pale greenish by humans). The ancestor of the lowland clade was reconstructed as bearing corollas with high reflectance in the overall short-, medium-, and long-wavelength ranges (from 400 up to 700 nm) and absorbing in the UV region (perceived as white by humans) while the ancestor of the Andean clade was reconstructed as bearing corollas with low overall reflectance (perceived as maroon by humans). Reconstruction of pollination mode in the MRCA of extant *Jaborosa* was ambiguous. The MRCA of the lowland clade was reconstructed as being mostly pollinated by hawkmoths about 4.2 Ma while the MRCA of the Andean clade was reconstructed as being pollinated by saprophilous flies about 2.8 Ma. Thus, pollination mode (hawkmoths vs. saprophilous flies) is contingent with changes in the corolla spectral shape. Finally, generalized pollination in *J. reflexa* appears to have evolved from a specialized saprophilous fly-pollinated ancestor (**Figure 4**).

Floral Color Discrimination in Pollinator Visual Spaces

Floral color loci in the *M. sexta* visual space for the three hawkmoth-pollinated species were clustered together in the region between blue and green photoreceptors and distant from the central zone, such that they would be perceived as colorful objects against the leaf background in the color space of *M. sexta* (**Figure 5A** and **Supplementary Figure 1**). The remaining *Jaborosa* species clustered around the central zone, such that they would be perceived by hawkmoths as colors very similar to the leaf background.

We detected a significant discrimination in corolla color between pollination modes in the visual space of *M. sexta*. The highest chromatic contrast was observed between hawkmoth- and saprophilous fly-pollinated species and between hawkmoth-pollinated species and the generalist-pollinated *J. reflexa*. No differences in chromatic contrast were detected among species pollinated by saprophilous flies and the generalist species (**Figure 5B**). When considering pairwise discrimination between *Jaborosa* species, chromatic distances were small and, in general, not significantly different within species sharing the same pollination mode but were significantly larger among species differing in pollination mode (**Figure 5C**). Thus, our results suggest a conservatism or convergence in corolla coloration within pollination modes. With regard to achromatic conspicuousness for hawkmoths, all achromatic contrasts among pollination modes were higher than 0.1 and thus were easily discriminable (**Supplementary Figure 2**).

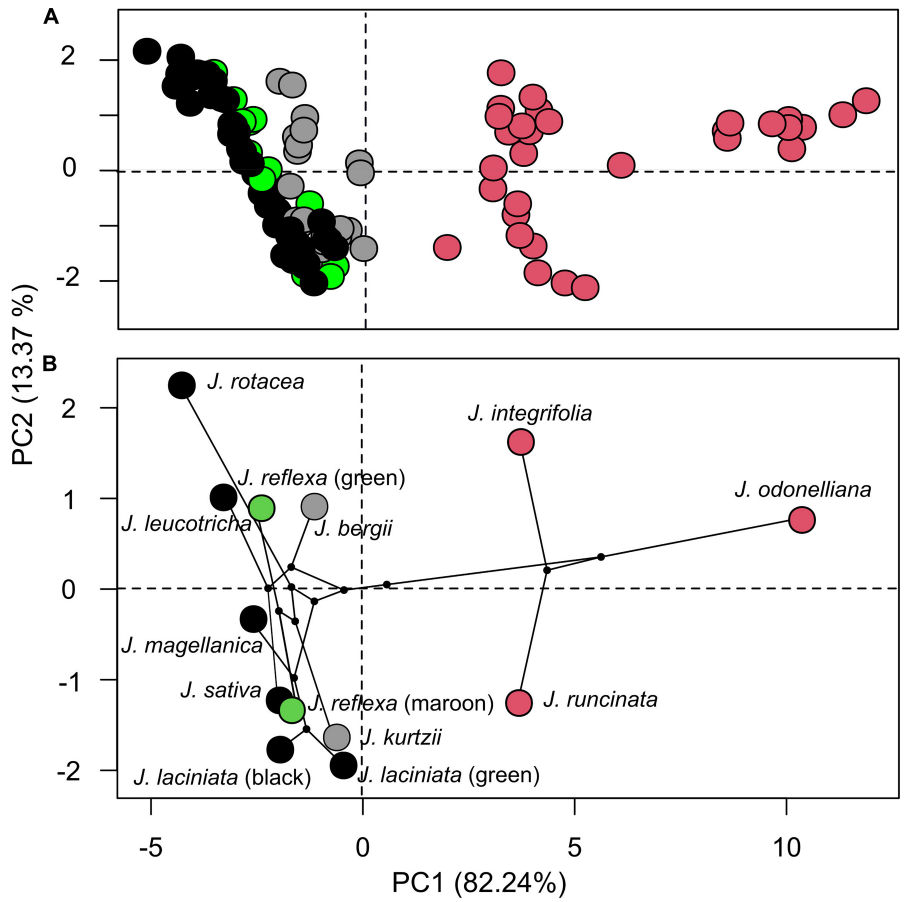


FIGURE 3 | Overall variation in corolla reflectance among *Jaborosa* species. **(A)** Spectral shape scatter plot from a PCA analysis, each point represents an individual. **(B)** Projection of the *Jaborosa* phylogenetic tree into the color space defined by the first two axes, each point represents the species mean. Different colors show pollination mode: pink for hawkmoths, black for saprophilous flies, green for generalized, and gray for unknown pollinators, respectively. Question marks in *J. laciniata* green morph and *J. reflexa* maroon morph stand for unknown pollinators.

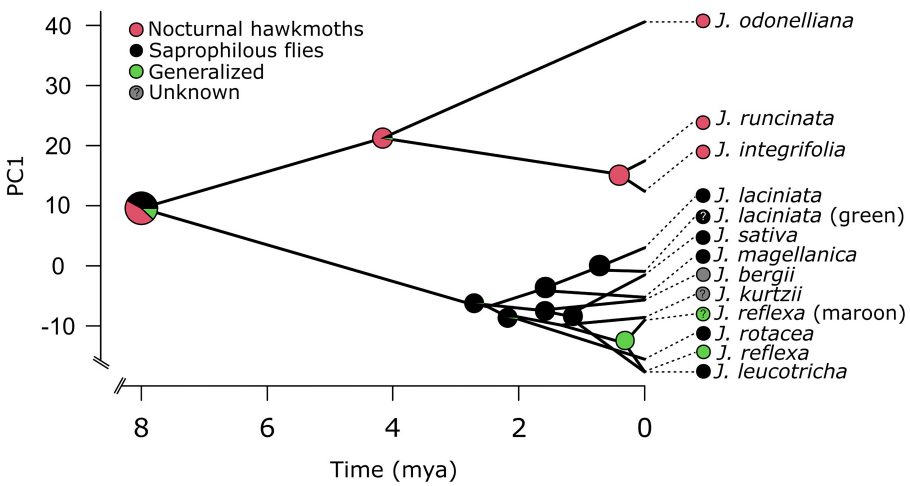


FIGURE 4 | Reconstruction of ancestral corolla color (PC1 loading scores from a standardized PCA analysis) and pollination mode in the genus *Jaborosa*. The phylogenetic tree was projected in the space defined by corolla spectral shape (*y*-axis) and divergence time (*x*-axis). Pie charts on each node show the posterior probability of each pollination mode retrieved by 100 stochastic character mappings. Question marks in *J. laciniata* green morph and *J. reflexa* maroon morph stand for unknown pollinators.

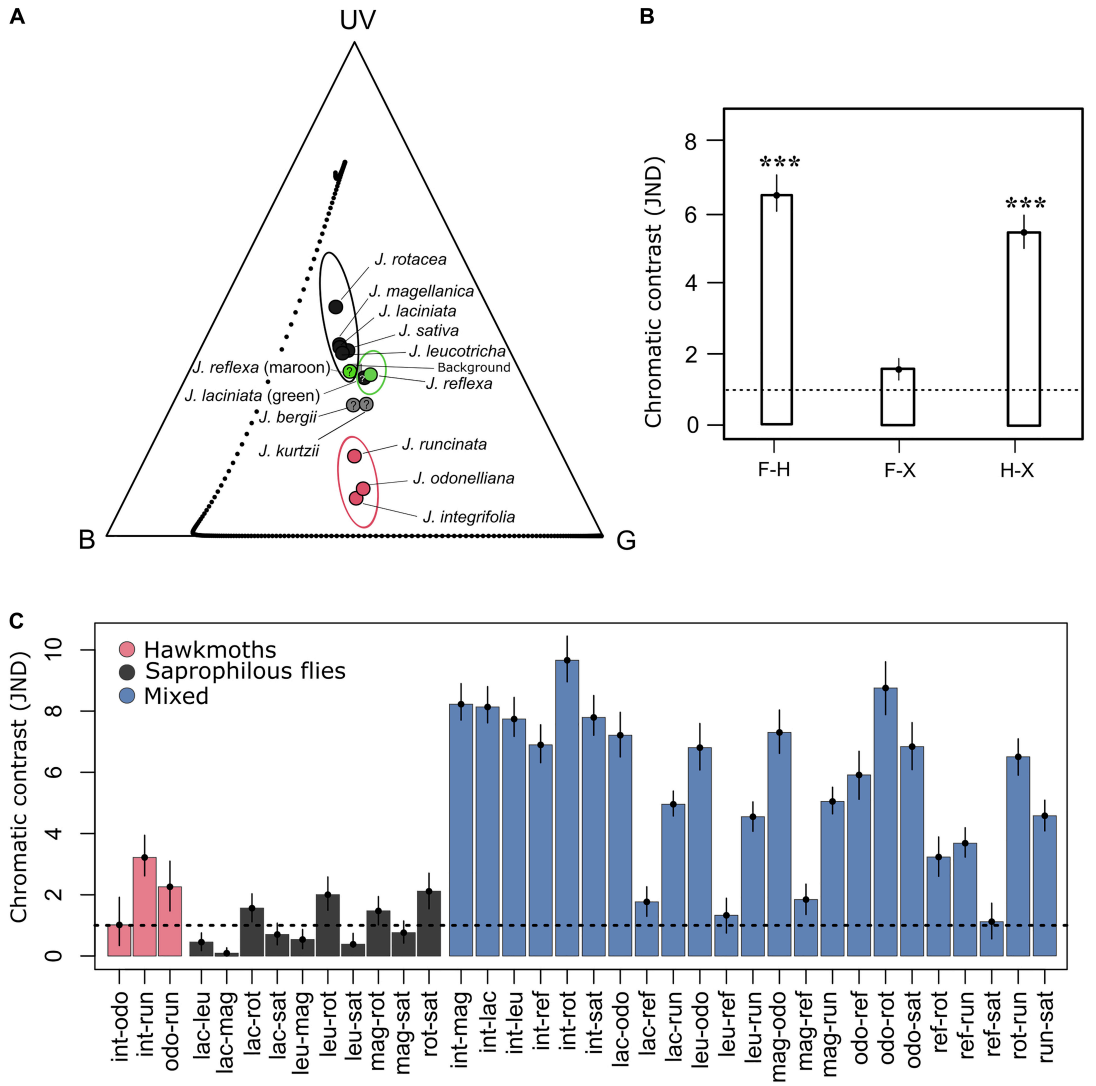


FIGURE 5 | Color loci of *Jaborosa* flowers in the trichromatic model of *Manduca sexta* and the bootstrapped, noise-corrected chromatic distances among *Jaborosa* species and pollination modes. **(A)** The vertices of the Maxwell triangle represent colors that excite only one of the three receptor types (UV, B and G). **(B)** Bars show mean chromatic distances among pollination modes. Whiskers show 95% confidence intervals for color discrimination (F, flies; H, hawkmoths; X, generalized pollination by small insects). The dashed line represents the theoretical discrimination threshold of one JND. ***Show a significant distance at $P < 0.001$ (PERMANOVA test). **(C)** Bars show mean chromatic distances between pairs of *Jaborosa* species ordered according to their pollination mode (pink for hawkmoth pollinated, gray for saprophilous fly-pollinated, blue for hawkmoth- vs. saprophilous fly-pollinated, hawkmoth vs. generalist and saprophilous fly-pollinated vs. generalist).

Floral color loci for the saprophilous fly-pollinated *Jaborosa* species all fall within the fly-UV quadrant of the visual spaces for the blowfly *Lucilia* sp. and the hoverfly *E. tenax* (**Figure 6A** and **Supplementary Figure 1**). Thus, neither carrion-flies nor hoverflies would be able to distinguish among *Jaborosa* species located in the same quadrant. In contrast, hawkmoth-pollinated species together with the green morph of *J. reflexa* – pollinated by a mixed array of pollinators-, *J. bergii* and the green morph of *J. laciniata* are placed within the fly-green quadrant (**Figure 6A** and **Supplementary Figure 1**).

We detected a significant discrimination in corolla color between pollination modes in the visual space of *Lucilia* sp. The highest Euclidean distance was observed between saprophilous fly- and hawkmoth-pollinated species, followed by the distances between the generalist-pollinated *J. reflexa* and hawkmoth-pollinated or saprophilous fly-pollinated *Jaborosa* species (**Figure 6B**). When considering pairwise discrimination between *Jaborosa* species, Euclidean distances were small among species sharing pollination mode (i.e., hawkmoths or saprophilous flies) but were significantly larger among species differing in pollination mode, given that most of them fell within different quadrants of blowfly visual space (**Figure 6C**).

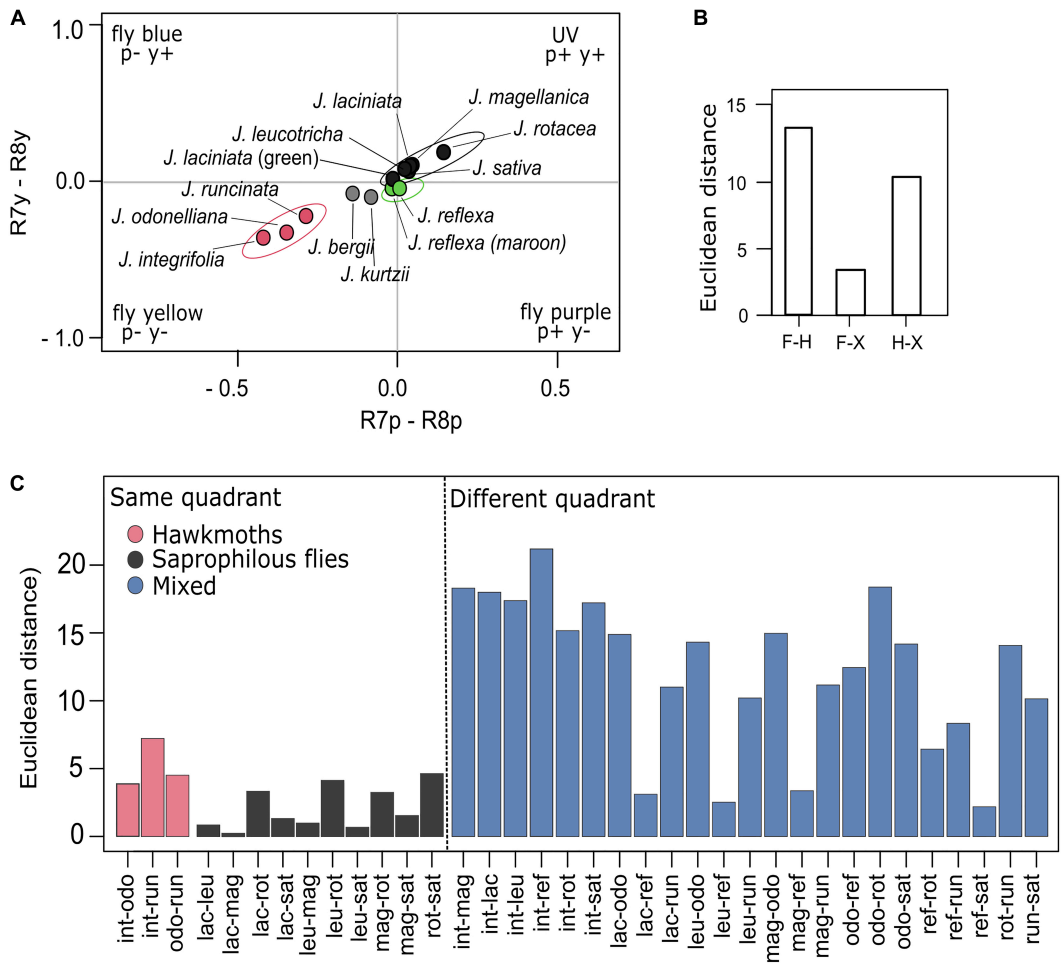


FIGURE 6 | Colors of *Jaborosa* flowers according to how they would be perceived by a blowfly. **(A)** COC for *Lucilia* sp. Colors in the same quadrant are not discriminated as different by the fly. **(B)** Bars show mean color distances (Euclidean distances) among pollination modes (F: Flies, H: Hawkmoths, X: Generalized pollination by small insects). **(C)** Bars show mean color distances (Euclidean distances) between pairs of *Jaborosa* species with known pollinators. Species are ordered according to their pollination mode (pink for hawkmoth pollinated, gray for saprophilous fly-pollinated, and blue for hawkmoth- vs. saprophilous fly-pollinated, hawkmoth vs. generalist and saprophilous fly-pollinated vs. generalist). The dotted vertical line separates those *Jaborosa* species pairs that are in the same quadrant (left) from the ones that are in different quadrants (right).

Evolution of Color Phenotype Across Environments

Evolutionary transitions along the color vision axes were significantly associated with the ordered transitions in environmental occupancy across the phylogeny of the studied *Jaborosa* species. This was true both for hawkmoth and fly visual model vectors (**Supplementary Table 3**, **Figure 7**, and **Supplementary Figure 3**).

DISCUSSION

Our results revealed a striking, non-random pattern of flower coloration associated with two primary modes of pollination in the genus *Jaborosa*: unrewarding (brood-site deceptive) flowers with inconspicuous and low reflective corollas mainly pollinated by saprophilous flies vs. nectar-rich flowers with conspicuous and highly reflective corollas pollinated by nocturnal hawkmoths. These utterly different pollination modes are separated geographically, as saprophilous fly pollination is associated with species growing in high altitude (Andes) or latitude (Patagonian steppe) environments, whereas nocturnal pollination by hawkmoths is associated with species growing in humid subtropical lowlands. Species with intermediate coloration patterns showed generalist pollination by small insects including saprophilous flies or unknown pollinators and are found in intermediate geographic locations such as dry lowland and foothill zones (**Figure 1**).

Flower Conspicuousness and Pollination Mode in *Jaborosa*

Similar patterns of corolla reflectance to those found here for sphingophilous *Jaborosa* species (i.e., absorbing in the UV

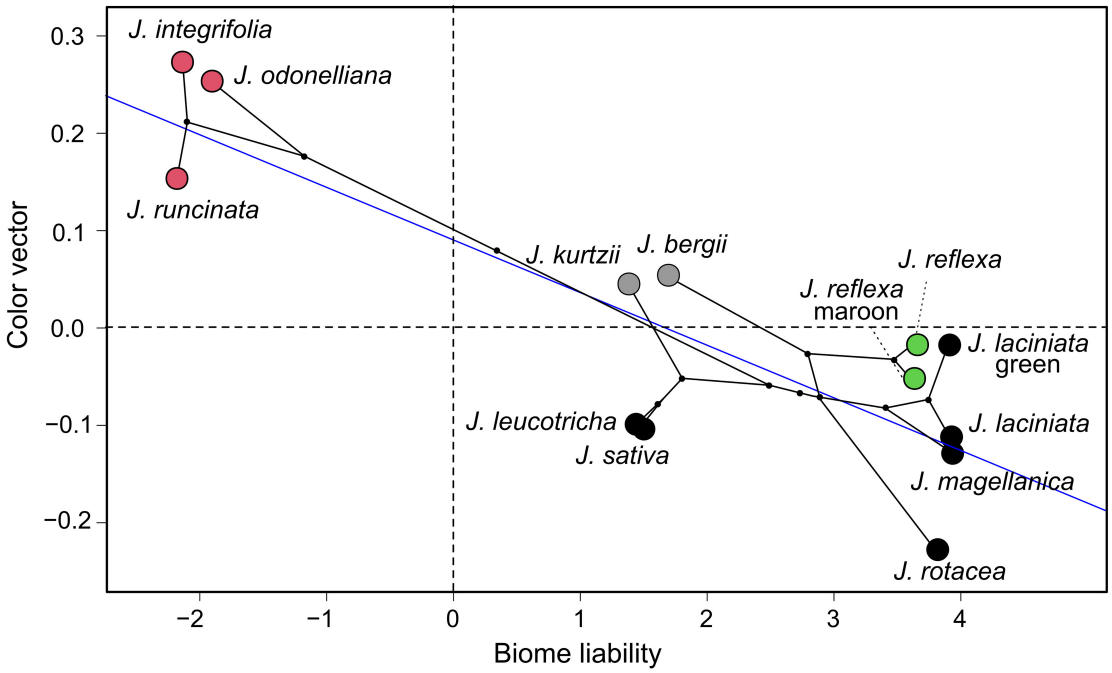


FIGURE 7 | Evolutionary transitions along the color vision axis. Phylogenetic generalized least squares correlation (PGLS) between environment occupancy (represented as liabilities, *x*-axis) and perceptual color vector of the nocturnal hawkmoth *Manduca sexta*. Blue line represents the slope. Different colors show pollination mode: pink for hawkmoths, black for saprophilous flies, green for multiple, and gray for unknown pollinators, respectively. See text for further explanations.

region and being highly reflective between 400 and 700 nm) were reported for other hawkmoth-pollinated plant species across the Americas, where nocturnal hawkmoths are widely attracted to flowers that appear "white" to humans (Haber and Frankie, 1989; White et al., 1994; Raguso et al., 2003). Nocturnal hawkmoths are major pollinators in South America where they visit hundreds of flowers each night to fuel their high energetic demands by drinking nectar (Sazatornil et al., 2016). Some nocturnal hawkmoths can discriminate flower coloration at starlight intensities at which humans and honeybees are colorblind (Kelber et al., 2002) but they rely on both olfactory and visual cues to recognize, approach and feed from flowers (Raguso and Willis, 2005; Goyret et al., 2007). *M. sexta* also can utilize achromatic (corolla brightness) signals, above-ambient CO_2, humidity and mechanosensory cues (corolla morphology) to successfully drink nectar from flowers (Goyret and Raguso, 2006; Goyret, 2010; Goyret and Kelber, 2012; review by Stöckl and Kelber, 2019). It is known that innate feeding behavior is mediated by blue photoreceptors in the nocturnal hawkmoth *M. sexta* (Cutler et al., 1995), which is able to switch color preferences under different photic conditions (Kuenzinger et al., 2019) or after training experience (Goyret et al., 2008) suggesting that both sensory plasticity and color learning capabilities play important roles in flower-foraging behavior.

Similar patterns of corolla reflectance to those found here for saprophilous fly-pollinated *Jaborosa* species have been reported for other sapromyiophilous plant species across the world (Urru et al., 2011; Chen et al., 2015; Du Plessis et al., 2018). Forensic studies indicate that the coloration of dead animal bodies is dynamic as their carcasses decay. Dull red *livor mortis* is observed after several hours following death, when greenish discoloration becomes brownish or black (Chen et al., 2015). We measured the full range of these color changes in the corollas of *Jaborosa* species pollinated by saprophilous flies (**Figure 1**). Saprophilous flies use both olfactory and visual cues to find oviposition substrates (Wall and Fisher, 2001; Chen et al., 2015). We previously demonstrated that floral scent, specifically sulfur-containing volatile compounds, plays an important role as an attractant of saprophilous flies to flowers of *J. rotacea* and *J. laciniata* in natural settings (Moré et al., 2013, 2019).

Evolutionary studies often focus on the evolution of specialized pollination systems while very interesting patterns may emerge from the evolution of generalized pollination from specialized pollination (Waser et al., 1996; Johnson and Steiner, 2000). Interestingly, we found that the evolution of generalized pollination mode in *J. reflexa* is likely to have evolved from a specialist ancestor pollinated by saprophilous flies (**Figure 4**). One possible mechanism to explain the evolution of generalist pollination in *J. reflexa* is a geographic range extension since is the only *Jaborosa* species growing in some areas of the vast Patagonian steppe. This species showed an interesting pattern of intra-specific variation in flower phenotype that merits further study.

Floral color conspicuousness (in terms of chromatic and achromatic contrast against the background; Schmidt et al., 2004) may influence pollinators' detection of *Jaborosa* flowers either for nectar foraging (hawkmoths, small insect) or oviposition deceit (saprophilous flies). Difference in color conspicuousness was

high and significant between the sphingophilous *Jaborosa* species and the diurnal pollinated species (as expected for *M. sexta*; **Figure 5B** and **Supplementary Figure 3**), but was small and not significant between the diurnal pollinated species (saprophilous fly-pollinated vs. generalist by small insects), as expected for *Lucilia* sp. flies (**Figure 6B**). Significantly greater conspicuousness (chromatic and achromatic) in the hawkmoth-pollinated species ensures that flowers could be detected by hawkmoths even under starlight conditions.

When taking into account color conspicuousness differences between species pairs, it is interesting that within the sphingophilous clade the short-tubed *J. runcinata* is perceived as different from the other two long-tubed species – *J. integrifolia* and *J. odonelliana* – in the visual space of *M. sexta* (JND > 1; **Figure 1**). It is possible that character displacement in tube length and corolla coloration represent prezygotic barriers to pollen flow between these taxa. This is relevant because *J. runcinata* and *J. integrifolia* are sympatric at our lowland study sites, where plants with morphologically intermediate flowers can be found (unpublished data). A similar trend was observed in the nightshade genus *Iochroma* where taxa that occur in sympatry occupy a significantly larger volume of color space than those in allopatry, suggesting that competition among close relatives may commonly underlie floral divergence, especially in species-rich habitats where congeners frequently co-occur (Muchhala et al., 2014).

On the other hand, the smallest color conspicuousness differences were observed among saprophilous fly-pollinated species. These species, which cluster in the UV quadrant of the *Lucilia* sp. color space, would be indistinguishable by the flies, given the visual model used. Interestingly, this is the quadrant in which flies perceive the skin color of dead cattle in a stage of *livor mortis* (Chen et al., 2015), suggesting that convergence in flower color is the result of mimicry. It was revealed that variation in coloration between different parts of the inflorescence of *Amorphophallus konjac* is associated with differential attraction of saprophilic flies (Chen et al., 2015). This niche partitioning between groups of calyptrate flies (e.g., sarcophagids vs. calliphorids) was also reported in the orchid *Satyrium pumilum*, in this case by flower size and scent intensity rather than coloration pattern of the corolla (Van Der Niet et al., 2011). We suspect that similar niche partitioning is occurring in *Jaborosa*, given observed variation in flower coloration, size, morphology, and scent composition and intensity and the diversity of fly visitors (**Supplementary Table 1**).

Geological Events Underlying Pollination Climates in *Jaborosa*

Andean orogeny and the ecological changes that followed have promoted diversification in plant and animal lineages since the Early Miocene (Luebert and Weigend, 2014). Studies on the paleobotany and geology of South America suggest that up to half of the Central Andes uplift (i.e., a rise from 2000 m to the present 4000 m) has taken place during the last 10 Ma. Divergence of the MRCA of *Jaborosa* in the two main clades occurred approximately 8 mya ago during the late Miocene, when geological evidence suggests that the Andes were still relatively low (400–2500 m) and climate was warm and humid at southern latitudes (**Figure 4**). The split of these two major clades has strong geographical structure, suggesting early isolation of these two ancestral lineages (**Figure 1**).

Interestingly, there is paleo-environmental evidence supporting the existence of a geographic barrier at the time of early divergence in *Jaborosa*. Studies have shown that during the middle and late Miocene (ca. 10–17 mya) three successive Atlantic marine transgressions, informally known as the "Paranean Sea," resulted in a flooded area in southern South America possibly separating the ancestors of these lineages (Tambussi and Degrange, 2012; see Svensson et al., 2016 for a similar example in North America). At the same time, cold desert climate covered the plains east of the Southern Andes and gave way during the Late Miocene (*ca.* 8 Ma) to the Patagonian steppe, which in the present features near-alpine climatic conditions (see Barreda et al., 2008). Concomitant with Andean orogeny, glaciation began in the late Pliocene (*ca.* 3 Ma), placing the origins of alpine Andean vegetation at ca. 3–5 Ma, making it the youngest natural ecosystem of South America (Graham, 2009). During the Pliocene, the Puna-Altiplano Plateau rose from 2500 to 4000 m.a.s.l. (∼5 Ma to present; Barnes and Ehlers, 2009) and small dust particles were transported by dust storms from the Puna-Altiplano Plateau to the Pampas grasslands and deposited by rain (∼3 Ma to present; Gaiero et al., 2013).

Pollinators' Perceptual Context of Flower Color Diversification

Extensive evidence has demonstrated that the latitudinal and altitudinal distribution of floral syndromes is influenced by the distributions of their respective pollinator guilds (Ollerton et al., 2006). In this context, the Andes provide a unique geographic scenario in southern South America, under which several pollinator transitions have been recognized (Pérez et al., 2006; Smith et al., 2008; Schlumpberger et al., 2009; Strelin et al., 2017; Dellinger et al., 2019). Our findings suggest that floral diversification in the genus *Jaborosa* could have occurred in concert with a geographic shift from Subtropical lowlands (Chaco and Pampas grasslands) to cool-temperate regions with marked changes in daily temperatures (Monte and Prepuna deserts, high mountain Andes and Patagonian steppe) accompanied by geographical differences in pollinator availability, specifically reduced abundance of hawkmoths. The harsh environmental conditions in high elevation Andean habitats (low temperatures, short growing seasons, and strong winds) generally reduce the spectrum of possibilities for biotic pollination. Colonization of these new habitats with colder temperatures reduce visitation by long-tongued hawkmoths because they are endothermic insects that rarely fly above 1300 m in Subtropical Argentina (Moré et al., 2014). Hawkmoth diversity is higher in subtropical lowland areas where ambient temperatures at dusk are moderate, and where nocturnal hawkmoths constitute a substantial component of the pollinator fauna (Cruden et al., 1976; Haber and Frankie, 1989; Sazatornil et al., 2016). Out of 117 hawkmoth

species recorded from Argentina, only the short-tongued *Hyles euphorbiarum* (proboscis length shorter than 30 mm), is consistently recorded beyond 40° south latitude (Moré et al., 2014). Interestingly, other plant lineages with significant amounts of sphingophily, such as *Nicotiana* (Solanaceae) and the family Cactaceae, show the same geographic and altitudinal patterns described here (Goodspeed, 1954; Schlumpberger and Renner, 2012). It is worth mentioning that in addition to metabolic limits, other factors may be involved in the distribution of hawkmoth fauna. In South Africa, the long-tongued *Agrius convolvuli* and its associated plant guild are only distributed in the eastern subtropical regions and are absent from the western Cape region and the arid south-west (Johnson and Raguso, 2016). This appears to be a general pattern because the Cape fynbos vegetation lacks families such as Rubiaceae, Balsaminaceae, Vitaceae, Convolvulaceae, and Loganiaceae that comprise the larval food plants for many hawkmoths (Attie et al., 2010).

Interestingly, the converse situation is not observed, given that the geographic distribution of saprophilous flies is not limited by ambient temperature and metabolic energetics. Calyptrate flies can tolerate high temperatures and are broadly distributed across geographical domains, to the extent that some species (e.g., *Lucilia sericata*) are used as nearly universal indicators in forensic studies (Mulieri et al., 2010). This asymmetry in pollinator distribution, which defines pollinator climate for *Jaborosa*, is highlighted by a simple experiment in which volatile sulfides typical of saprophilous fly pollinated *J. laciniata* were added to the large, white flowers of *J. integrifolia* in its lowland habitat during daytime. Within minutes of scent augmentation, calliphorid flies approached, landed upon, and attempted to feed from the sphingophilous flowers of *J. integrifolia* (Moré et al., 2019). Thus, the putative direction of evolution in **Figure 4** (e.g., from a sphingophilous ancestry) suggests a transition to fly pollination in colder, drier habitats devoid of hawkmoths, whereas saprophilous flies are present in all habitats occupied by *Jaborosa* species.

Pollination by saprophilous flies becomes substantial in the high-mountain Andes where pollinator limitation strongly limits the success of self-incompatible plant species (Arroyo et al., 1982; Pérez et al., 2009). Particularly, the flesh-fly genus *Microcerella*, recorded here as pollinators of the Patagonian species *J. magellanica* and *J. reflexa*, show greater diversity in arid and high-altitude environments of South America (Mulieri and Mariluis, 2009). Saprophilous fly-pollinated species of *Jaborosa* are restricted to regions where species pollinated by long-tongued hawkmoths are absent (neither beyond 40° South latitude nor above 3000 m in the Andes. **Figure 1**). Although brood-site deceptive fly-pollinated species of *Jaborosa* are restricted to the such cold-temperate environments, other plant lineages pollinated or putatively pollinated by saprophilous flies are distributed in warm-temperate and subtropical areas of South America, such as *Aristolochia* spp. (Aristolochiaceae), *Synandrospadix vermitoxicus* (Araceae), *Pleurothallis* spp. (Orchidaceae) and *Gonolobus* spp. (Apocynaceae), and plants with similar reproductive strategies are found in warm/subtropical biomes worldwide (review by Jürgens et al., 2006; Urru et al., 2011; Jürgens et al., 2013).

CONCLUSION

Our results revealed two major evolutionary themes for the flower color diversification pattern in the South American genus *Jaborosa*. The first is a "warm subtropical sphingophilous clade" composed of three hawkmoth-pollinated species found in humid lowland habitats, with large white flowers that clustered together in the "blue-green" region and distant from the central zone in the visual space of the model hawkmoth *M. sexta* visual space. Thus, they are perceived as colorful objects against the vegetative background in hawkmoth color space (**Figure 5**). The second is a "cool-temperate brood-deceptive clade" composed of largely fly-pollinated species found at high altitudes (Andes) or latitudes (Patagonian Steppe), with small, dark flowers that clustered together in the UV quadrant in the visual space of the model blowfly *Lucilia* sp. (**Figure 6**). Our findings, based on multivariate analyses of reflectance spectra, ancestral reconstruction of flower color and pollination mode and comparative methods (**Figures 3**, **4**, **7**) suggest that the ability of plants to colonize newly formed environments during Andean orogeny and the ecological changes that followed were concomitant with adjustment in flower color as perceived by different pollinator groups. Adaptation to habitat and pollination mode are inextricably linked in the history of this South American plant lineage.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The flower reflectance spectra analyzed in this study will be available at the Floral Reflectance Database (ID #4383–#4505 at http://www.reflectance.co.uk/).

AUTHOR CONTRIBUTIONS

MM, AAC, and RAR contributed to the design and implementation of the research. MM, ACI, MED, and AAC contributed to the analysis of the results. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.601975/full#supplementary-material

Supplementary Figure 1 | Color loci of all measured *Jaborosa* individuals in the trichromatic model of the nocturnal hawkmoth *Manduca sexta* (upper panel) and how they would be perceived by the blowfly *Lucilia* sp. (bottom left) and the hoverfly *Eristalis tenax* (bottom right).

Supplementary Figure 2 | Mean differences in color conspicuousness (achromatic contrast) among pollination modes (F, flies; H, hawkmoths; X, generalized pollination by small insects). The dashed line represents the theoretical discrimination threshold of 0.1 JND.

Supplementary Figure 3 | Evolutionary transitions along the color vision axis. Phylogenetic generalized least squares correlation (PGLS) between environment occupancy and perceptual color vector of the blowfly *Lucilia* sp. (see text for further explanations). The dashed horizontal line (at −0.9 in the *y*-axis) corresponds to the center of the fly color space where all the quadrants coincide. Thus, *Jaborosa* species that are in the p−y− quadrant are shown above the line, and those *Jaborosa* species that are in the p+y+ quadrant are shown below the line.

Supplementary Table 1 | *Jaborosa* species studied for the corolla reflectance analyses and visual models. We provide information about pollinator observations (direct observation, ligh trapping and hawkmoths' scales analysis) in different localities. Asterisks show confirmed pollinators.

Supplementary Table 2 | Environmental zones classification used in the PGLS analyses: humid lowland zone (1), dry lowland and foothill zone (2), and alpine and high latitude zone (3).

Supplementary Table 3 | Phylogenetic generalized least squares model among *Jaborosa* species between environmental occupancy and flower color expressed in hawkmoth and blowfly vision models.

REFERENCES

Arnold, S. E. J., Savolainen, V., and Chittka, L. (2009). Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. *Arthropod Plant Interact.* 3, 27–43. doi: 10.1007/s11829-009-9056-9

Arroyo, M. T. K., Primack, R., and Armesto, J. (1982). Community studies in pollination ecology in the high temperate Andes of central Chile. I. Pollination mechanisms and altitudinal variation. *Am. J. Bot.* 69, 82–97. doi: 10.1002/j.1537-2197.1982.tb13237.x

Attie, M., Kitching, I. J., and Veslot, J. (2010). Patterns of larval hostplant usage among hawkmoths (Lepidoptera, Sphingidae) from La Réunion, with a comparison of the Mascarenes with other regions of the world. *Revue d'Ecologie* 65, 3–44.

Balkenius, A., Kelber, A., and Balkenius, C. (2004). A model of selection between stimulus and place strategy in a hawkmoth. *Adapt. Behav.* 12, 21–35. doi: 10.1177/105971230401200101

Barnes, J. B., and Ehlers, T. A. (2009). End member models for Andean Plateau uplift. *Earth Sci. Rev.* 97, 105–132. doi: 10.1016/j.earscirev.2009.08.003

Barreda, V., Guler, V., and Palazzesi, L. (2008). "Late Miocene continental and marine palynological assemblages from Patagonia," in *The Late Cenozoic of Patagonia and Tierra Del Fuego*, ed. J. Rabassa (Oxford: Elsevier), 343–349. doi: 10.1016/s1571-0866(07)10016-6

Bennett, R. R., and Brown, P. K. (1985). Properties of the visual pigments of the moth *Manduca sexta* and the effects of two detergents, digitonin and CHAPS. *Vision Res.* 25, 1771–1781. doi: 10.1016/0042-6989(85)90002-1

Bollback, J. P. (2006). SIMMAP: stochastic character mapping of discrete traits on phylogenies. *BMC Bioinform.* 7:88. doi: 10.1186/1471-2105-7-88

Bradshaw, H. D., and Shemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Chacoff, N. P., Vázquez, D. P., Lomáscolo, S. B., Stevani, E. L., Dorado, J., and Padrón, B. (2012). Evaluating sampling completeness in a desert plant–pollinator network. *J. Anim. Ecol.* 81, 190–200. doi: 10.1111/j.1365-2656.2011.01883.x

Chartier, M., Jabbour, F., Gerber, S., Mitteroecker, P., Sauquet, H., Von Balthazar, M., et al. (2014). The floral morphospace–a modern comparative approach to study angiosperm evolution. *New Phytol.* 204, 841–853. doi: 10.1111/nph.12969

Chaves, J. A., Weir, J. T., and Smith, T. B. (2011). Diversification in Adelomyia hummingbirds follows Andean uplift. *Mol. Ecol.* 20, 4564–4576. doi: 10.1111/j.1365-294X.2011.05304.x

Chen, G., Ma, X. K., Jürgens, A., Lu, J., Liu, E. X., Sun, W. B., et al. (2015). Mimicking livor mortis: a well-known but unsubstantiated color profile in sapromyiophily. *J. Chem. Ecol.* 41, 808–815. doi: 10.1007/s10886-015-0618-2

Chen, Z., Niu, Y., Liu, C. Q., and Sun, H. (2020). Red flowers differ in shades between pollination systems and across continents. *Ann. Bot.* 126, 837–848. doi: 10.1093/aob/mcaa103

Chittka, L., and Kevan, P. G. (2005). "Flower colors as advertisement," in *Practical Pollination Biology*, eds A. Dafni and P. G. Kevan (Cambridge, ON: Enviroquest Ltd.), 157–206.

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/BF00188925

Cruden, R. W., Kinsman, S., Stockhouse, R. E., and Linhart, Y. B. (1976). Pollination, fecundity, and the distribution of moth-flowered plants. *Biotropica* 8, 204–210. doi: 10.2307/2989686

Cuthill, I. C., Bennett, A. T., Partridge, J. C., and Maier, E. J. (1999). Plumage reflectance and the objective assessment of avian sexual dichromatism. *Am. Nat.* 153, 183–200. doi: 10.1086/303160

Cutler, D., Bennett, R., Stevenson, R., and White, R. (1995). Feeding behavior in the nocturnal moth *Manduca sexta* is mediated mainly by blue receptors, but where are they located in the retina? *J. Exp. Biol.* 198, 1909–1917.

De Ibarra, N. H., and Vorobyev, M. (2009). Flower patterns are adapted for detection by bees. *J. Comp. Physiol. A* 195, 319–323. doi: 10.1007/s00359-009-0412-0

Dellinger, A. S., Scheer, L. M., Artuso, S., Fernández-Fernández, D., Sornoza, F., Penneys, D. S., et al. (2019). Bimodal pollination systems in Andean Melastomataceae involving birds, bats, and rodents. *Am. Nat.* 194, 104–116. doi: 10.1086/703517

Devoto, M., Medan, D., and Montaldo, N. H. (2005). Patterns of interaction between plants and pollinators along an environmental gradient. *Oikos* 109, 461–472. doi: 10.1111/j.0030-1299.2005.13712.x

Drummond, A. J., Suchard, M. A., Xie, D., and Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* 29, 1969–1973. doi: 10.1093/molbev/mss075

Du Plessis, M., Johnson, S. D., Nicolson, S. W., Bruyns, P. V., and Shuttleworth, A. (2018). Pollination of the "carrion flowers" of an African stapeliad (*Ceropegia*

mixta: Apocynaceae): the importance of visual and scent traits for the attraction of flies. *Plant Syst. Evol.* 304, 357–372. doi: 10.1007/s00606-017-1481-0

Elberling, H., and Olesen, J. M. (1999). The structure of a high latitude plant−flower visitor system: the dominance of flies. *Ecography* 22, 314–323. doi: 10.1111/j.1600-0587.1999.tb00507.x

Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.3732/ajb.0800222

Felsenstein, J. (1973). Maximum-likelihood estimation of evolutionary trees from continuous characters. *Am. J. Hum. Genet.* 25, 471–492.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Gaiero, D. M., Simonella, L., Gassó, S., Gili, S., Stein, A. F., Sosa, P., et al. (2013). Ground/satellite observations and atmospheric modeling of dust storms originating in the high Puna-Altiplano deserts (South America): implications for the interpretation of paleo-climatic archives. *J. Geophys. Res. Atmos.* 118, 3817–3831. doi: 10.1002/jgrd.50036

Goodspeed, T. H. (1954). *The Genus Nicotiana*. Waltham, MA: Chronica Botanica Company, 1–536.

Goyret, J. (2010). Look and touch: multimodal sensory control of flower inspection movements in the nocturnal hawkmoth *Manduca sexta*. *J. Exp. Biol.* 213, 3676–3682. doi: 10.1242/jeb.045831

Goyret, J., and Kelber, A. (2012). Chromatic signals control proboscis movements during hovering flight in the hummingbird hawkmoth *Macroglossum stellatarum*. *PLoS One* 7:e34629. doi: 10.1371/journal.pone.0034629

Goyret, J., and Raguso, R. A. (2006). The role of mechanosensory input in flower handling efficiency and learning by *Manduca sexta*. *J. Exp. Biol.* 209, 1585–1593. doi: 10.1242/jeb.02169

Goyret, J., Markwell, P. M., and Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *J. Exp. Biol.* 210, 1398–1405. doi: 10.1242/jeb.02752

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Sci. Nat.* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Graham, A. (2009). The Andes: a geological overview from a biological perspective. *Ann. Mo. Bot. Gard.* 96, 371–385. doi: 10.3417/2007146

Grant, V. (1994). Modes and origins of mechanical and ethological isolation in angiosperms. *Proc. Natl. Acad. Sci. U.S.A.* 91, 3–10. doi: 10.1073/pnas.91.1.3

Grant, V., and Grant, K. A. (1965). *Flower Pollination in the Phlox family*. New York, NY: Columbia University Press.

Gutierrez De Camargo, M. G., Lunau, K., Batalha, M. A., Brings, S., De Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Haber, W. A., and Frankie, G. W. (1989). A tropical hawkmoth community: Costa Rican dry forest Sphingidae. *Biotropica* 21, 155–172. doi: 10.2307/2388706

Hannah, L., Dyer, A. G., Garcia, J. E., Dorin, A., and Burd, M. (2019). Psychophysics of the hoverfly: categorical or continuous color discrimination? *Curr. Zool.* 65, 483–492. doi: 10.1093/cz/zoz008

Hansen, T. F. (1997). Stabilizing selection and the comparative analysis of adaptation. *Evolution* 51, 1341–1351. doi: 10.1111/j.1558-5646.1997.tb01457.x

Hardie, R. C., and Kirschfeld, K. (1983). Ultraviolet sensitivity of fly photoreceptors R7 and R8: evidence for a sensitising function. *Biophys. Struct. Mech.* 9, 171–180. doi: 10.1007/bf00537814

Harmon, L. J., Losos, J. B., Jonathan Davies, T., Gillespie, R. G., Gittleman, J. L., Bryan Jennings, W., et al. (2010). Early bursts of body size and shape evolution are rare in comparative data. *Evolution* 64, 2385–2396. doi: 10.1111/j.1558-5646.2010.01025.x

Harmon, L. J., Weir, J. T., Brock, C. D., Glor, R. E., and Challenger, W. (2008). GEIGER: investigating evolutionary radiations. *Bioinformatics* 24, 129–131. doi: 10.1093/bioinformatics/btm538

Henze, M. J., Lind, O., Mappes, J., Rojas, B., and Kelber, A. (2018). An aposematic colour–polymorphic moth seen through the eyes of conspecifics and predators–sensitivity and colour discrimination in a tiger moth. *Funct. Ecol.* 32, 1797–1809. doi: 10.1111/1365-2435.13100

Hodges, S. A., Fulton, M., Yang, J. Y., and Whittall, J. B. (2004). Verne Grant and evolutionary studies of *Aquilegia*. *New Phytol.* 161, 113–120. doi: 10.1046/j.1469-8137.2003.00950.x

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-96582003084[1733:TROHIT]2.0.CO;2

Johnsen, S., Kelber, A., Warrant, E., Sweeney, A. M., Widder, E. A., Lee, R. L., et al. (2006). Crepuscular and nocturnal illumination and its effects on color perception by the nocturnal hawkmoth *Deilephila* elpenor. *J. Exp. Biol.* 209, 789–800. doi: 10.1242/jeb.02053

Johnson, S. D. (2010). The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 499–516. doi: 10.1098/rstb.2009.0243

Johnson, S. D., and Raguso, R. A. (2016). The long-tongued hawkmoth pollinator niche for native and invasive plants in Africa. *Ann. Bot.* 117, 25–36. doi: 10.1093/aob/mcv137

Johnson, S. D., and Steiner, K. E. (2000). Generalization versus specialization in plant pollination systems. *Trends Ecol. Evol.* 15, 140–143. doi: 10.1016/s0169-5347(99)01811-x

Johnson, S. D., Moré, M., Amorim, F. W., Haber, W. A., Frankie, G. W., Stanley, D. A., et al. (2017). The long and the short of it: a global analysis of hawkmoth pollination niches and interaction networks. *Funct. Ecol.* 31, 101–115. doi: 10.1111/1365-2435.12753

Jürgens, A., Dötterl, S., and Meve, U. (2006). The chemical nature of fetid floral odours in stapeliads (Apocynaceae-Asclepiadoideae-Ceropegieae). *New Phytol.* 172, 452–468. doi: 10.1111/j.1469-8137.2006.01845.x

Jürgens, A., Wee, S. L., Shuttleworth, A., and Johnson, S. D. (2013). Chemical mimicry of insect oviposition sites: a global analysis of convergence in angiosperms. *Ecol. Lett.* 16, 1157–1167. doi: 10.1111/ele.12152

Kay, K. M., Reeves, P. A., Olmstead, R. G., and Schemske, D. W. (2005). Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *Am. J. Bot.* 92, 1899–1910. doi: 10.3732/ajb.92.11.1899

Kelber, A., Balkenius, A., and Warrant, E. J. (2002). Scotopic colour vision in nocturnal hawkmoths. *Nature* 419, 922–925. doi: 10.1038/nature01065

Kuenzinger, W., Kelber, A., Weesner, J., Travis, J., Raguso, R. A., and Goyret, J. (2019). Innate colour preferences of a hawkmoth depend on visual context. *Biol. Lett.* 15:20180886. doi: 10.1098/rsbl.2018.0886

Luebert, F., and Weigend, M. (2014). Phylogenetic insights into Andean plant diversification. *Front. Ecol. Evol.* 2:27. doi: 10.3389/fevo.2014.00027

Lunau, K. (2014). Visual ecology of flies with particular reference to colour vision and colour preferences. *J. Comp. Physiol. A* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Maia, R., and White, T. E. (2018). Comparing colors using visual models. *Behav. Ecol.* 29, 649–659. doi: 10.1093/beheco/ary017

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Moré, M., Cocucci, A. A., and Raguso, R. A. (2013). The importance of oligosulfides in the attraction of fly pollinators to the brood-site deceptive species *Jaborosa* rotacea (Solanaceae). *Int. J. Plant Sci.* 174, 863–876. doi: 10.1086/670367

Moré, M., Cocucci, A. A., Sérsic, A. N., and Barboza, G. E. (2015). Phylogeny and floral trait evolution in *Jaborosa* (Solanaceae). *Taxon* 64, 523–534. doi: 10.12705/643.8

Moré, M., Kitching, I. J., and Cocucci, A. A. (2014). "Sphingidae," in Biodiversidad de Artrópodos Argentinos, Vol. 4, eds S. Roig-Juñent, L. E. Claps, and J. J. Morrone (San Miguel de Tucumán, AR), 281-295.

Moré, M., Mulieri, P., Battán-Horenstein, M., Cocucci, A. A., and Raguso, R. A. (2019). The role of fetid olfactory signals in the shift to saprophilous fly pollination in *Jaborosa* (Solanaceae). *Arthropod Plant Interact.* 13, 375–386. doi: 10.1007/s11829-018-9640

Moré, M., Sérsic, A. N., and Cocucci, A. A. (2006). Specialized use of pollen vectors by *Caesalpinia* gilliesii, a legume species with brush-type flowers. *Biol. J. Linn. Soc. Lond.* 88, 579–592. doi: 10.1111/j.1095-8312.2006.00644.x

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Mulieri, P. R., and Mariluis, J. C. (2009). New species and new records of Microcerella Macquart (Diptera: Sarcophagidae) belonging to the *M. spinigena* species-group. *Neotrop. Entomol.* 38, 101–103. doi: 10.1590/S1519-566X2009000100010

Mulieri, P. R., Mariluis, J. C., and Patitucci, L. D. (2010). Review of the Sarcophaginae (Diptera: Sarcophagidae) of Buenos Aires Province (Argentina), with a key and description of a new species. *Zootaxa* 2575, 1–37. doi: 10.11646/zootaxa.2575.1.1

Ollerton, J., Johnson, S. D., Hingston, A. B., Waser, N. M., and Ollerton, J. (2006). *Geographical Variation in Diversity and Speci?city of Pollination Systems*. Chicago, IL: University of Chicago Press, 283–308.

Olson, D. M., Dinerstein, E., Wikramanayake, E. D., Burgess, N. D., Powell, G. V., Underwood, E. C., et al. (2001). Terrestrial ecoregions of the world: a new map of life on earth: a new global map of terrestrial ecoregions provides an innovative tool for conserving biodiversity. *BioScience* 51, 933–938. doi: 10.1641/0006-35682001051[0933:TEOTWA]2.0.CO;2

Paine, K. C., White, T. E., and Whitney, K. D. (2019). Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints? *Evol. Ecol.* 33, 461–479. doi: 10.1007/s10682-019-09991-2

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20, 289–290. doi: 10.1093/bioinformatics/btg412

Pérez, F., Arroyo, M. T. K., and Armesto, J. J. (2009). Evolution of autonomous selfing accompanies increased specialization in the pollination system of *Schizanthus* (Solanaceae). *Am. J. Bot.* 96, 1168–1176. doi: 10.3732/ajb.0800306

Pérez, F., Arroyo, M. T., Medel, R., and Hershkovitz, M. A. (2006). Ancestral reconstruction of flower morphology and pollination systems in *Schizanthus* (Solanaceae). *Am. J. Bot.* 93, 1029–1038. doi: 10.3732/ajb.93.7.1029

Phillips, R. D., Peakall, R., van der Niet, T., and Johnson, S. D. (2020). Niche perspectives on plant–pollinator interactions. *Trends Plant Sci.* 25, 779–793. doi: 10.1016/j.tplants.2020.03.009

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raguso, R. A., and Willis, M. A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Anim. Behav.* 69, 407–418. doi: 10.1016/j.anbehav.2004.04.015

Raguso, R. A., Henzel, C., Buchmann, S. L., and Nabhan, G. P. (2003). Trumpet flowers of the Sonoran Desert: floral biology of *Peniocereus* cacti and sacred *Datura*. *Int. J. Plant Sci.* 164, 877–892. doi: 10.1086/378539

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Kelber, A., and Schaefer, H. M. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Revell, L. J. (2014). Ancestral character estimation under the threshold model from quantitative genetics. *Evolution* 68, 743–759. doi: 10.1111/evo.12300

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., et al. (2014). A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.* 17, 388–400. doi: 10.1111/ele.12224

Salzmann, U., Haywood, A. M., Lunt, D. J., Valdes, P. J., and Hill, D. J. (2008). A new global biome reconstruction and data-model comparison for the middle Pliocene. *Glob. Ecol. Biogeogr.* 17, 432–447. doi: 10.1111/j.1466-8238.2008.00381.x

Sazatornil, F. D., More, M., Benitez-Vieyra, S., Cocucci, A. A., Kitching, I. J., Schlumpberger, B. O., et al. (2016). Beyond neutral and forbidden links: morphological matches and the assembly of mutualistic hawkmoth–plant networks. *J. Anim. Ecol.* 85, 1586–1594. doi: 10.1111/1365-2656.12509

Schaefer, H. M., and Ruxton, G. D. (2011). *Plant-Animal Communication*. Oxford: OUP Oxford.

Schlumpberger, B. O., and Renner, S. S. (2012). Molecular phylogenetics of *Echinopsis* (Cactaceae): polyphyly at all levels and convergent evolution of pollination modes and growth forms. *Am. J. Bot.* 99, 1335–1349. doi: 10.3732/ajb.1100288

Schlumpberger, B. O., Cocucci, A. A., Moré, M., Sérsic, A. N., and Raguso, R. A. (2009). Extreme variation in floral characters and its consequences for pollinator attraction among populations of an Andean cactus. *Ann. Bot.* 103, 1489–1500. doi: 10.1093/aob/mcp075

Schmidt, V., Martin Schaefer, H., and Winkler, H. (2004). Conspicuousness, not colour as foraging cue in plant–animal signalling. *Oikos* 106, 551–557. doi: 10.1111/j.0030-1299.2004.12769.x

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Smith, S. D., Ané, C., and Baum, D. A. (2008). The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution* 62, 793–806. doi: 10.1111/j.1558-5646.2008.00327.x

Stöckl, A. L., and Kelber, A. (2019). Fuelling on the wing: sensory ecology of hawkmoth foraging. *J. Comp. Physiol. A* 205, 399–413. doi: 10.1007/s00359-019-01328-2

Streisfeld, M. A., and Kohn, J. R. (2007). Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evol. Biol.* 20, 122–132. doi: 10.1111/j.1420-9101.2006.01216.x

Strelin, M. M., Arroyo, J. I., Fliesswasser, S., and Ackermann, M. (2017). Diversification of *Caiophora* (Loasaceae subfam. Loasoideae) during the uplift of the Central Andes. *Org. Divers. Evol.* 17, 29–41. doi: 10.1007/s13127-016-0312-4

Svensson, G. P., Raguso, R. A., Flatz, R., and Smith, C. I. (2016). Floral scent of Joshua trees (*Yucca brevifolia* sensu lato): divergence in scent profiles between species but breakdown of signal integrity in a narrow hybrid zone. *Am. J. Bot.* 103, 1793–1802. doi: 10.3732/ajb.1600033

Tambussi, C. P., and Degrange, F. (2012). *South American and Antarctic Continental Cenozoic Birds: Paleobiogeographic Affinities and Disparities*. Berlin: Springer Science & Business Media.

Telles, F. J., Kelber, A., and Rodríguez-Gironés, M. A. (2016). Wavelength discrimination in the hummingbird hawkmoth *Macroglossum stellatarum*. *J. Exp. Biol.* 219, 553–560. doi: 10.1242/jeb.130484

Telles, F. J., Lind, O., Henze, M. J., Rodríguez-Gironés, M. A., Goyret, J., and Kelber, A. (2014). Out of the blue: the spectral sensitivity of hummingbird hawkmoths. *J. Comp. Physiol. A* 200, 537–546. doi: 10.1007/s00359-014-0888-0

Troje, N. (1993). Spectral categories in the learning behaviour of blowflies. *Z. Naturforsch. C* 48, 96–104. doi: 10.1515/znc-1993-1-218

Urru, I., Stensmyr, M. C., and Hansson, B. S. (2011). Pollination by brood-site deception. *Phytochemistry* 72, 1655–1666. doi: 10.1016/j.phytochem.2011.02.014

Van Der Niet, T., Hansen, D. M., and Johnson, S. D. (2011). Carrion mimicry in a South African orchid: flowers attract a narrow subset of the fly assemblage on animal carcasses. *Ann. Bot.* 107, 981–992. doi: 10.1093/aob/mcr048

Van Der Niet, T., Peakall, R., and Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Ann. Bot.* 113, 199–212. doi: 10.1093/aob/mct290

Vesprini, J. L., and Galetto, L. (2000). The reproductive biology of *Jaborosa integrifolia* (Solanaceae): why its fruits are so rare? *Plant. Syst. Evol.* 225, 15–28. doi: 10.1007/BF00985456

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. B* 265, 351–358. doi: 10.1098/rspb.1998.0302

Wall, R., and Fisher, P. (2001). Visual and olfactory cue interaction in resource location by the blowfly, *Lucilla sericata*. *Physiol. Entomol.* 26, 212–218. doi: 10.1046/j.0307-6962.2001.00237.x

Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., and Ollerton, J. (1996). Generalization in pollination systems, and why it matters. *Ecology* 77, 1043–1060. doi: 10.2307/2265575

White, R. H., Stevenson, R. D., Bennett, R. R., Cutler, D. E., and Haber, W. A. (1994). Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. *Biotropica* 26, 427–435. doi: 10.2307/2389237

White, R. H., Xu, H., Münch, T. A., Bennett, R. R., and Grable, E. A. (2003). The retina of *Manduca sexta*: rhodopsin expression, the mosaic of green-, blue-and UV-sensitive photoreceptors, and regional specialization. *J. Exp. Biol.* 206, 3337–3348. doi: 10.1242/jeb.00571

Whittall, J. B., and Hodges, S. A. (2007). Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447, 706–710. doi: 10.1038/nature05857

Wright, S. (1934). An analysis of variability in number of digits in an inbred strain of guinea pigs. *Genetics* 19, 506–536.

Yamashiro, T., Yamashiro, A., Yokoyama, J., and Maki, M. (2008). Morphological aspects and phylogenetic analyses of pollination systems in the *Tylophora–Vincetoxicum* complex (Apocynaceae-Asclepiadoideae) in Japan. *Biol. J. Linn. Soc.* 93, 325–341. doi: 10.1111/j.1095-8312.2007.00896.x

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Chemical Basis of Floral Color Signals in Gesneriaceae: The Effect of Alternative Anthocyanin Pathways

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Changes in floral pigmentation can have dramatic effects on angiosperm evolution by making flowers either attractive or inconspicuous to different pollinator groups. Flower color largely depends on the type and abundance of pigments produced in the petals, but it is still unclear whether similar color signals rely on same biosynthetic pathways and to which extent the activation of certain pathways influences the course of floral color evolution. To address these questions, we investigated the physical and chemical aspects of floral color in the Neotropical Gesnerioideae (ca. 1,200 spp.), in which two types of anthocyanins, hydroxyanthocyanins, and deoxyanthocyanins, have been recorded as floral pigments. Using spectrophotometry, we measured flower reflectance for over 150 species representing different clades and pollination syndromes. We analyzed these reflectance data to estimate how the Gesnerioideae flowers are perceived by bees and hummingbirds using the visual system models of these pollinators. Floral anthocyanins were further identified using high performance liquid chromatography coupled to mass spectrometry. We found that orange/red floral colors in Gesnerioideae are produced either by deoxyanthocyanins (e.g., apigenidin, luteolinidin) or hydroxyanthocyanins (e.g., pelargonidin). The presence of deoxyanthocyanins in several lineages suggests that the activation of the deoxyanthocyanin pathway has evolved multiple times in the Gesnerioideae. The hydroxyanthocyanin-producing flowers span a wide range of colors, which enables them to be discriminated by hummingbirds or bees. By contrast, color diversity among the deoxyanthocyanin-producing species is lower and mainly represented at longer wavelengths, which is in line with the hue discrimination optima for hummingbirds. These results indicate that Gesnerioideae have evolved two different biochemical mechanisms to generate orange/red flowers, which is associated with hummingbird pollination. Our findings also suggest that the activation of the deoxyanthocyanin pathway has restricted flower color diversification to orange/red hues, supporting the potential constraining role of this alternative biosynthetic pathway on the evolutionary outcome of phenotypical and ecological diversification.

Keywords: Gesneriaceae, anthocyanin pathway, deoxyanthocyanin, visual systems, hummingbird pollination, floral pigments, chromatic signal

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INTRODUCTION

Flower color plays a key role in angiosperm reproduction by attracting pollinators. Floral color acts as a visual cue for pollinators to associate certain visual signals with reward (Chittka and Menzel, 1992; Schiestl and Johnson, 2013). Not all pollinators have the same color vision, and different pollinator groups perceive and select flowers differently. Bees have trichromatic vision with photoreceptors sensitive to UV, blue, and green, which enables them to detect short- to medium-wavelengths (Chittka, 1992). On the other hand, flower-visiting birds, such as hummingbirds, have a tetrachromatic color vision with photoreceptors sensitive to UV, blue, green, and red, which enables them to detect not only short- and medium-, but also long-wavelengths (Herrera et al., 2008; Stoddard et al., 2020). Adaptations to these differences in pollinator visual processing have been considered as a major driver of floral color diversification in angiosperms and the repeated evolution of color signals specific to certain functional pollinator groups. For instance, bee-pollinated flowers tend to have reflectance between 400 and 500 nm wavelengths, which matches the discrimination optima for bees (von Helversen, 1972; Dyer et al., 2012). On the other hand, bird-pollinated flowers frequently have a distinctive orange/red color, which is better detected by birds than by bees especially against a green vegetation background (Lunau et al., 2011; Shrestha et al., 2013; Burd et al., 2014; Bergamo et al., 2016). Despite the importance of color signaling in plant-pollinator interactions, the biochemical basis of color shifts has been investigated in a limited number of studies (Hoballah et al., 2007; Sobel and Streisfeld, 2013; Ng and Smith, 2016), and our understanding of how pigment biosynthesis pathways shaped the evolution of floral color is still limited (Cronk and Ojeda, 2008; Rausher, 2008; Ng et al., 2018; Wheeler and Smith, 2019).

As one of the most common floral pigment groups, anthocyanins are water-soluble flavonoid compounds that produce a broad range of hues including blue, purple, pink, orange, and red (Tanaka et al., 2008; Zhao and Tao, 2015). The anthocyanin biosynthesis pathway (ABP; **Figure 1**) involves several structural and regulatory proteins that orchestrate the production of a wide array of pigments derived from three 3-hydroxylated anthocyanidin precursors: pelargonidin, cyanidin, and delphinidin (Holton and Cornish, 1995; Albert et al., 2014). In addition to these 3-hydroxyanthocyanins, the ABP can also produce 3-deoxyanthocyanins (e.g., apigenidin, luteolinidin), which lack the hydroxyl group on carbon 3 and are derived from an alternative branch of the ABP that uses flavanone as a substrate (Winefield et al., 2005; Grotewold, 2006; Piatkowski et al., 2020). Unlike 3-hydroxyanthocyanins, 3-deoxyanthocyanins produce limited hues ranging from orange to red and they are much less common in plants. These uncommon anthocyanin pigments have only been detected in ferns, mosses, few members of Poaceae (in sorghum, maize, and sugarcane) and Theaceae (tea), and some New World clades of Bignoniaceae and Gesneriaceae so far (Harborne, 1966, 1967; Winefield et al., 2005; Piatkowski et al., 2020). While the biochemical production of floral anthocyanins has been extensively studied, the relative importance of deoxyanthocyanins in floral color and its detection by pollinators still needs to be evaluated.

Gesnerioideae is the Neotropical subfamily of Gesneriaceae, and it consists of 75 genera with over 1,200 species (Perret et al., 2013). The outstanding floral color diversity, the presence of both hydroxy- and deoxy-anthocyanins pigments, and several transitions between hummingbird and bee pollination syndromes throughout the evolution of this clade make Gesnerioideae an appropriate study system to examine the biochemical mechanisms underlying floral color transitions and their impact on plant-pollinator interactions (Roberts and Roalson, 2017, 2020; Serrano-Serrano et al., 2017). Based on the presence of two alternative branches of the ABP in the Gesnerioideae, we predict that (i) the deoxyanthocyanin-producing plants have an additional biochemical solution to produce orange/red flowers, and that (ii) these plants are limited to producing orange/red hues, which results in reduced floral color diversity and a higher degree of specialization on hummingbird pollination when compared to the hydroxyanthocyanin-producing lineages. In this study, we aim to test these predictions by evaluating the relative contributions of the hydroxyanthocyanin and deoxyanthocyanin branches of the ABP to the evolution of orange/red flowers in the Gesnerioideae. To achieve this, we identified the anthocyanin compositions and measured reflectance spectra of hummingbird- and bee-pollinated flowers representing 156 species from different Gesnerioideae lineages. We also used hummingbird and bee visual models to evaluate how anthocyanin composition affects flower detection by pollinators. Our results will contribute to a better understanding of the chemical basis of color signals and how alternative branches of the ABP may affect floral color diversification.

MATERIALS AND METHODS

Sampling

We collected pigment and reflectance data from 180 samples representing 156 Gesnerioideae species, exhibiting the full range of floral color diversity and pollination syndromes within this Neotropical clade (**Supplementary Table S1**). Flowers were collected from plants cultivated at the Botanical Garden of Geneva (Switzerland), in Mauro Peixoto private collection (Sao Paulo, Brazil), and during field trips in Brazil, Colombia, and Ecuador (see **Supplementary Table S1** for voucher information). Observation-based pollinator information was available for 56 species (see **Supplementary Table S2** for references).

Detection and Identification of Anthocyanin Pigments

Anthocyanin pigments were extracted from fresh or silica-dried corollas using methanol:HCl (99.9:0.1, v/v). Anthocyanin profiling was performed by ultra-high performance liquid chromatography-diode array detection-quadrupole time-of-flight mass spectrometry (UHPLC-DAD-QTOFMS). The UHPLC system was an Acquity UPLC (Waters, Milford, MA), the DAD detector an eλ PDA (Waters), and the QTOFMS

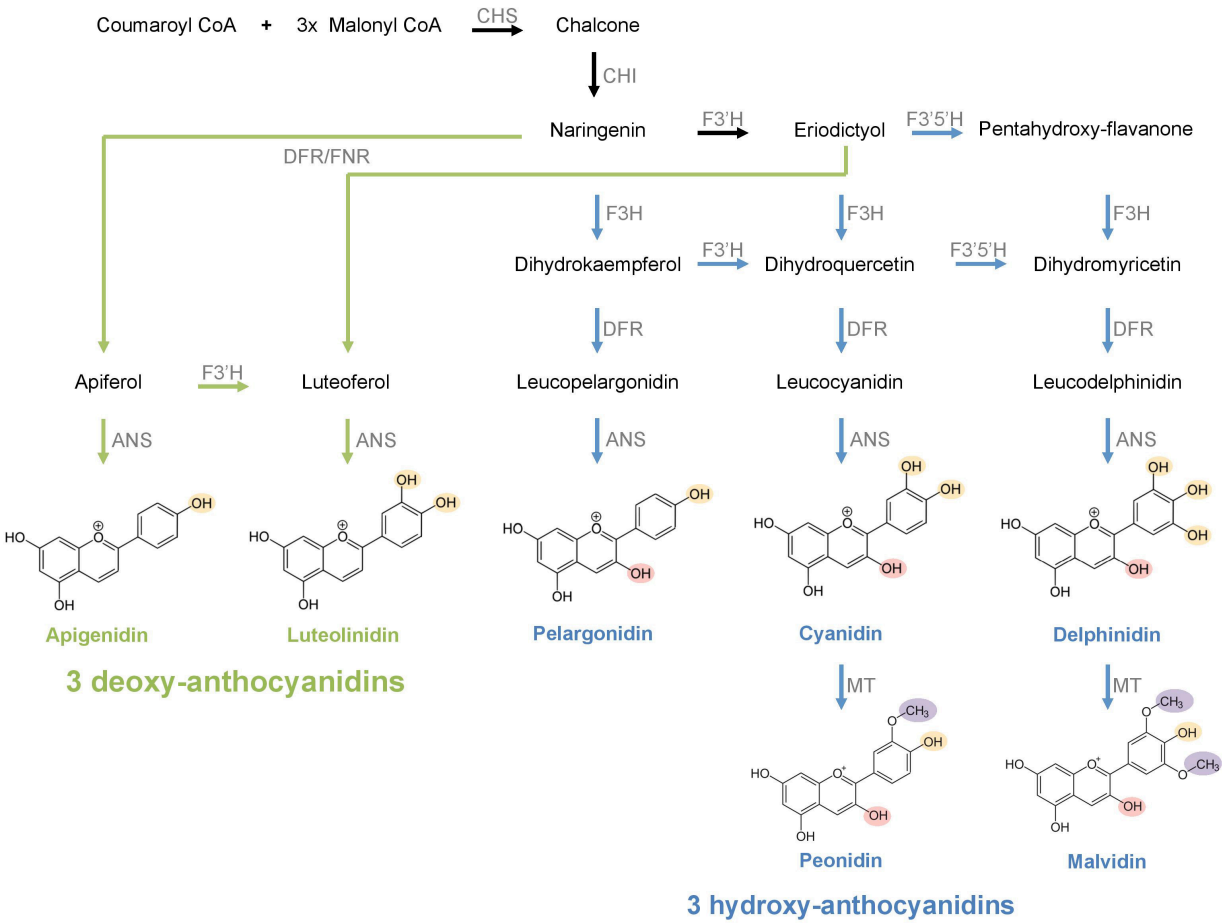


FIGURE 1 | The anthocyanin biosynthesis pathway. The reactions leading to hydroxy- and deoxy-anthocyanin compounds are represented in blue and green arrows, respectively. Earlier reactions preceding the two alternative pathways are represented in black arrows. OH groups differing between hydroxy- and deoxy-anthocyanins are colored in pink. OH groups in varying numbers in different anthocyanidins are colored in yellow. CH_3 groups in different hydroxy-anthocyanins are colored in purple. CHS, chalcone synthase; CHI, chalcone isomerase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; FNR, flavone synthase; ANS, anthocyanidin synthase; MT, methyltransferase.

a Synapt G2 (Waters). The entire system was controlled by Masslynx 4.1 (Waters). One microliter of extract was injected on an Acquity UPLC CSH C18 column (100 × 2.1 mm i.d., 1.7 µm particle size) maintained at 45°C. Mobile phases consisted of H_2O supplemented with formic acid 0.15% (phase A) and acetonitrile supplemented with formic acid 0.05% (phase B). The flow rate was of 0.4 mL/min and the following gradient was applied: 2–30% phase B in 6 min, 30–100% phase B in 2 min, hold at 100% phase B for 2 min, and re-equilibration at 2% phase B for 4 min. The DAD parameters were set as follows: range 190–600 nm, resolution 1.2 nm, sampling rate 20 Hz, time constant 0.1 s. The mass spectrometer was operated in electrospray positive ionization over a mass range of 85–1,200 Da. The so-called MSe mode was used, in which two acquisition functions are set in parallel: a low fragmentation (collision energy 4 eV) function yielding ions of the molecular species, and a high fragmentation (ramp 10–30 eV) function yielding fragments of interest. Accurate mass measurements were obtained by infusing a solution of the synthetic peptide leucine-enkephalin (m/z 556.2771 Da) in the mass spectrometer. The detection and identification of anthocyanins were performed by (1) selecting all peaks absorbing between 480 and 530 nm in the DAD chromatogram and reporting absorbance spectra and maxima; (2) extracting the corresponding peaks in the low fragmentation mass spectrum and computing the most probable molecular formulae; and (3) interpreting the aglycone and the sequence of sugars from the high fragmentation mass spectrum. The Dictionary of Natural Products[1] was further used to identify the detected anthocyanins. These anthocyanins were integrated in the software TargetLynx™ (Waters) and quantified using calibration curves built from standard solutions of luteolinidin, apigenidin and pelargonidin-3-rutinoside at 0.2, 1, 5, 20, and 100 µg/mL.

To simplify the chemistry data for downstream analyses, we divided the samples into four categories. "DEO90" and "HYD90" categories represent samples with more than

[1]http://dnp.chemnetbase.com

90% deoxyanthocyanin and hydroxyanthocyanin pigments, respectively. The third category is labeled as "DEO+HYD" and it includes samples that produce between 10 and 90% of both pigments. The fourth category includes samples with no anthocyanin.

A principal component analysis (PCA) biplot was generated using the R packages ade4 (Dray and Dufour, 2007) and factoextra (Kassambara and Mundt, 2020). The anthocyanin compositions were coded as continuous variables based on the relative percentages of the different anthocyanidins present in each sample. In order to account for the phylogenetic signal, phylogenetic PCA was also performed using two correlation structure methods (Brownian motion and Pagel's lambda with maximum likelihood optimization) implemented in the R package phytools (Revell, 2012).

Quantification of Floral Color

We quantified floral color on fresh corollas by measuring spectral reflectance for wavelengths between 300 and 700 nm using a portable JAZ spectrometer with a UV-VIS light source (Ocean Optics, Inc., Dunedin, FL). We calibrated the spectrometer using a Spectralon reflectance standard WS-1 (Ocean Optics, Inc., Dunedin, FL), and performed the measurements at a 45° angle with the probe shielding from any ambient light to avoid background noise. We measured one to three flowers per sample at up to three different points on the corollas. For flowers with uniform coloration or minor colored patterns (e.g., small spots or stripes), all measurements were averaged to obtain a single spectrum for each sample. For flowers with different lobe and tube colors, measurements for each part were averaged separately, resulting in two spectra per sample. We processed the reflectance data on the R package PAVO (Maia et al., 2013, 2019), and generated reflectance plots using LOESS smoothing with a smoothness parameter of 0.25.

We followed the method described by Chittka et al. (1994) to classify flower color. We divided the color spectrum into four regions: (i) 300–400 nm as UV; (ii) 400–500 nm as blue; (iii) 500–600 nm as green; and (iv) 600–700 nm as red. The region was coded "+" if the reflectance values within that region were above 10%, and coded "−" if below 10%. When the reflectance values above 10% were measured near the upper-boundary of the region, the region was coded as "/." Based on this floral color classification, the UV-b-g-r+ and UV-b-g/r+ flowers were coded as "orange/red," and the other flowers were coded as "other colors."

Ancestral State Reconstruction

The evolution of deoxyanthocyanin production was reconstructed on the Gesnerioideae phylogeny from Serrano-Serrano et al. (2017), after having pruned the tree to only include the 156 study species. Maximum likelihood-based ancestral reconstruction was performed using Markov-k state 1 model implemented in Mesquite 3.03 (Maddison and Maddison, 2014). The presence and absence of anthocyanin production were coded as 1 and 0, respectively. The percentage of deoxyanthocyanin present in each sample was mapped onto the Gesnerioideae phylogeny using the method anc.ML (Ancestral character estimation using likelihood) under the function *contMap* (Map continuous trait evolution on the tree) implemented the R package phytools (Revell, 2012). As outgroup, we selected Didymocarpoideae, which was previously shown to produce no deoxyanthocyanins (Harborne, 1966, 1967).

Visual Models for Bees and Hummingbirds

In order to visualize how different pollinators perceive floral colors, we used standard color space models for bees and hummingbirds that are included in the *vismodel* function of the R package PAVO (Maia et al., 2013, 2019). For bee vision, we used the visual mode "*Apis mellifera*," and we set the longest-wavelength photoreceptor as achromatic, and green foliage as background. We visualized the samples on the two-dimensional hexagonal color space that is used for trichromatic visual systems (Chittka, 1992). For hummingbird vision, we used the visual mode "*average avian V system*" and set green foliage as background. We visualized the samples on the three-dimensional tetrahedral color space that is used for tetrachromatic visual systems (Herrera et al., 2008).

For the two-dimensional bee visual space, we calculated the areas occupied by "DEO90" and "HYD90" samples using the function *mcp* (minimum convex polygon) implemented in the R package adehabitatHR (Calenge, 2006). In order to test the hypothesis that the DEO90 samples occupy a smaller area than a subset of samples randomly selected from the whole dataset, we performed 100 random subsampling and obtained confidence intervals for the area occupied by the random subsets. Random subsampling was performed using the RAND function on Microsoft Excel and the area calculations of the subsets were performed using the *mcp* function in the R package adehabitatHR (Calenge, 2006) as described above. Confidence intervals and the *p*-values were calculated using the R package gmodels (Warnes, 2005).

Estimation of the Color Discrimination Ability of Pollinators

A color is best discriminated by a visual system if there is a rapid change in the reflectance values along the spectrum where the sensitivities of different photoreceptors overlap (Chittka and Menzel, 1992). To estimate how well the flowers are discriminated by bee and hummingbird visual systems, we quantified the location of these reflectance changes on the spectrum by calculating the middle point of a steep curve (marker points), where the change was higher than 20%, as previously described (Chittka and Menzel, 1992; Dyer et al., 2012; Shrestha et al., 2013). We used the function *peakshape* in the R package PAVO (Maia et al., 2013, 2019) to calculate the marker points for each sample. Depending on the shape of the reflectance curve, the total number of marker points per sample ranged from one to three (**Supplementary Table S1**).

We quantified the match between marker points and the color discrimination optima for bees and hummingbirds using two metrics described in Shrestha et al. (2013). Color discrimination optima for visual systems of bees (400 and 500 nm) and

hummingbirds (460, 540, and 600 nm) were obtained from previous studies (von Helversen, 1972; Emmerton and Delius, 1980). The first metric used in this method is the mean absolute deviation (MAD), which is calculated as the average absolute difference between each marker point and the closest color discrimination optimum of a vision system. The second metric, minimum absolute deviation (minAD), considers each visual optimum separately and is obtained by calculating the absolute distance of the closest marker point to the visual optimum. For both MAD and minAD, small and large values represent high and low discrimination abilities, respectively.

We used phylogenetic ANOVA within the R package phytools (Revell, 2012) and the Gesnerioideae phylogeny (Serrano-Serrano et al., 2017) to test the differences in the MAD_Bee and MAD_Hummingbird values between the DEO-dominant (>50% deoxyanthocyanidin) and HYD-dominant (>50% hydroxyanthocyanidin) flowers. We performed 10,000 simulations and used Holm-Bonferroni method to adjust *p*-values for multiple comparisons.

RESULTS

Anthocyanin Identification and Composition in the Gesnerioideae

According to HPLC retention times, UV-vis absorption spectral characteristics, and high-resolution MS/MS spectra, we identified 12 anthocyanins (nine hydroxyanthocyanins and three deoxyanthocyanins) from the 180 samples (**Supplementary Tables S1, S3**). The aglycone parts of these 12 compounds correspond to five hydroxyanthocyanidins (pelargonidin, cyanidin, peonidin, malvidin, and delphinidin), and three deoxyanthocyanidins (apigenidin, luteolinidin, and an unidentified deoxy-anthocyanin pigment). We detected no anthocyanins in 28 samples. In the remaining 152 samples, pelargonidin (in 84 samples), cyanidin (52), malvidin (40), and luteolinidin (39) were the four most commonly produced anthocyanins. The anthocyanin composition was variable among the samples: 35.6% of the samples had a single anthocyanidin type, whereas the remaining samples produce a mix of two (27.2%), three (13.9%), or four to six different anthocyanidins (7.8%). Some anthocyanidin combinations were more frequently observed than the others (**Supplementary Figure S1**). The three most common combinations were (i) pelargonidin and cyanidin/peonidin (in 36 samples); (ii) cyanidin/peonidin and delphinidin/malvidin (33); and (iii) pelargonidin and delphinidin/malvidin (27). Apigenidin was the least common anthocyanidin, and it was only observed in combination with other anthocyanidins.

The PCA based on the relative frequencies of each anthocyanidin showed that sampled flowers are clustered according to their anthocyanidin composition (**Figure 2**). The first two principal components explained the 70.1% of the variation within the dataset, while the addition of the third principal component increased this percentage to 99.4% (**Supplementary Figure S2**). PC1 mainly reflected the abundance of the orange/red anthocyanidins (pelargonidin and the deoxyanthocyanins) versus other anthocyanidins. PC2 roughly corresponded to the presence of deoxyanthocyanins versus hydroxyanthocyanins. We observed that orange/red flowers were mostly clustered on right side of the plot, linked to the pelargonidin and deoxyanthocyanidin loadings, but we observed some orange/red flowers that were linked to the cyanidin/peonidin and delphinidin/malvinidin loadings. These results demonstrate that both hydroxy- and deoxy-anthocyanins contribute to the production of orange/red flowers in the Gesnerioideae. The results of the phylogenetic PCA were almost identical to the regular PCA under both Brownian motion and Pagel's lambda models ($\lambda = 0.000066$), indicating that the effect of phylogenetic signal on the clustering of samples was negligible.

The Evolution of Deoxyanthocyanin Production

Deoxyanthocyanins were identified in four subtribes and were particularly common in the genera *Columnea, Glossoloma, Nematanthus*, and *Sinningia* (**Figure 3** and **Supplementary Table S1**). The di-hydroxylated luteolinidin-5-glucoside was detected dominantly in the genus *Nematanthus*, whereas the mono-hydroxylated apigenidin-5-glucoside was mainly restricted to the genus *Sinningia* suggesting that the regulation of deoxyanthocyanin production may differ among clades. The ancestral state reconstruction analysis showed that the activation of the deoxyanthocyanin pathway has evolved (and occasionally been lost) several times throughout the evolutionary history of the Gesnerioideae (**Figure 3**). These independent evolutions of deoxyanthocyanin production suggest convergent evolution of this anthocyanin type within the Gesnerioideae.

The Color Diversity of the Gesnerioideae Flowers

Based on the previously established floral color classification (Chittka et al., 1994), we observed 9 different color groups within the Gesnerioideae (**Figure 4**). The UV-b-g-r+ and UV-b-g/r+ groups were coded as orange/red flowers and they constitute 50% (90 out of 180) of the studied samples. 25.6% of the orange/red flowers were DEO90, 31.1% were DEO+HYD, and 43.3% was HYD90. The rest of the color groups, which included white, cream, yellow, purple, pink, and green flowers, the majority of them were HYD90 (63.3%), 31.1% had no anthocyanins, and very few were DEO90 (1.1%) or DEO+HYD (4.4%). These non-orange/red DEO90 and DEO+HYD flowers have pink or brown hues, which may seem orange/red to human eye, but they are not categorized as "orange/red" according to our color classification.

We observed that the HYD90 samples occupy a larger space than the DEO90 samples in both hexagonal (bee) and tetrahedral (hummingbird) color spaces (**Figure 5**). Subsampling analysis of 100 random subsets suggests that the space occupied by the DEO90 samples is significantly smaller than any space occupied by random subsets ($p < 0.0001$).

We observed some intraspecific variation in our dataset. In many cases, species with multiple samples were highly similar in terms of both floral color and pigment composition (e.g., *Drymonia serrulata, Sinningia speciosa*). However,

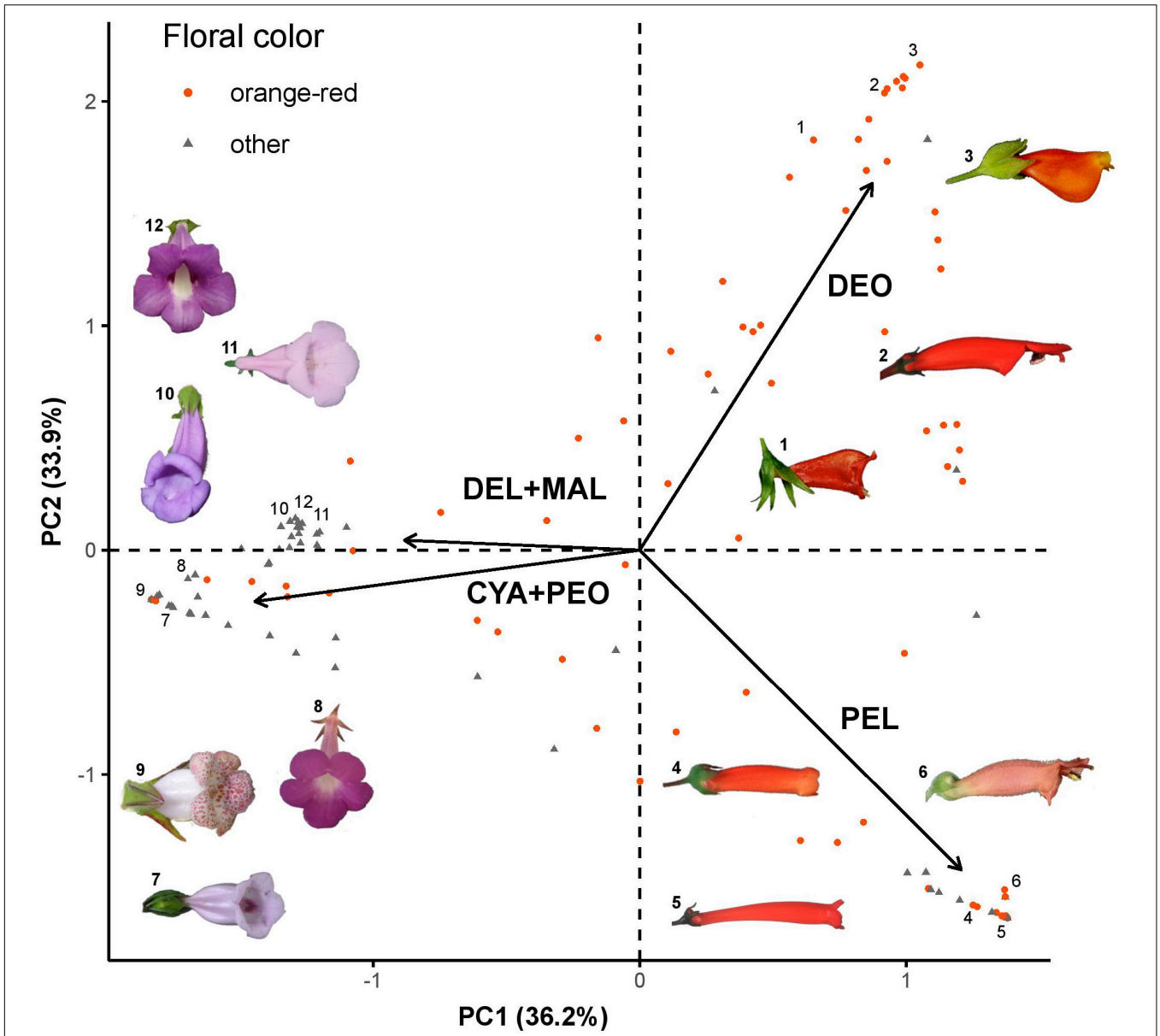


FIGURE 2 | PCA plot of Gesnerioideae flowers based on their anthocyanin composition. Arrows represent 4 loadings: PEL, pelargonidin; CYA+PEO, cyanidin and peonidin; DEL+MAL, delphinidin and malvinidin; DEO, deoxyanthocyanin (luteolinidin and apigenidin). Orange/red flowers are shown as orange dots, flowers with other colors are shown as gray triangles. 1: *Nematanthus crassifolius*, 2: *Sinningia glaziovana*, 3: *N. strigillosus*, 4: *S. aggregata*, 5: *S. gigantifolia*, 6: *S. elatior*, 7: *Paliavana tenuiflora*, 8: *Achimenes grandiflora*, 9: *N. whieleri*, 10: *S. sp. nov.1*, 11: *S. eumorpha*, and 12: *S. speciosa*.

few species with multiple samples showed some variation in pigment compositions (e.g., *Glossoloma ichthyoderma*, *Nematanthus crassifolius*), or floral color (e.g., *Kohleria spicata*, *Nematanthus fornix*).

Effect of the Anthocyanin Type on Floral Color Discrimination by Pollinators

To investigate the visual capacities of bees and hummingbirds, we compared the difference in the marker point distribution among the samples (**Figure 6**). We found that marker points for the DEO90 and DEO+HYD samples concentrated around 600 nm, where the long-wavelength sensitivity of hummingbird vision is highest. However, the marker points for the HYD90 samples were concentrated not only around 600 nm, but also around 400 and 500 nm, where the short- and medium-length wavelength sensitivity of bee-vision is highest. In other words, whereas DEO90 and DEO+HYD flowers better match the hummingbird visual system, HYD90 flowers match the visual systems of both pollinators.

Concordant with the marker point distribution patterns, the values for MAD_Bee (p = 0.001) and MAD_bird (p = 0.0001)

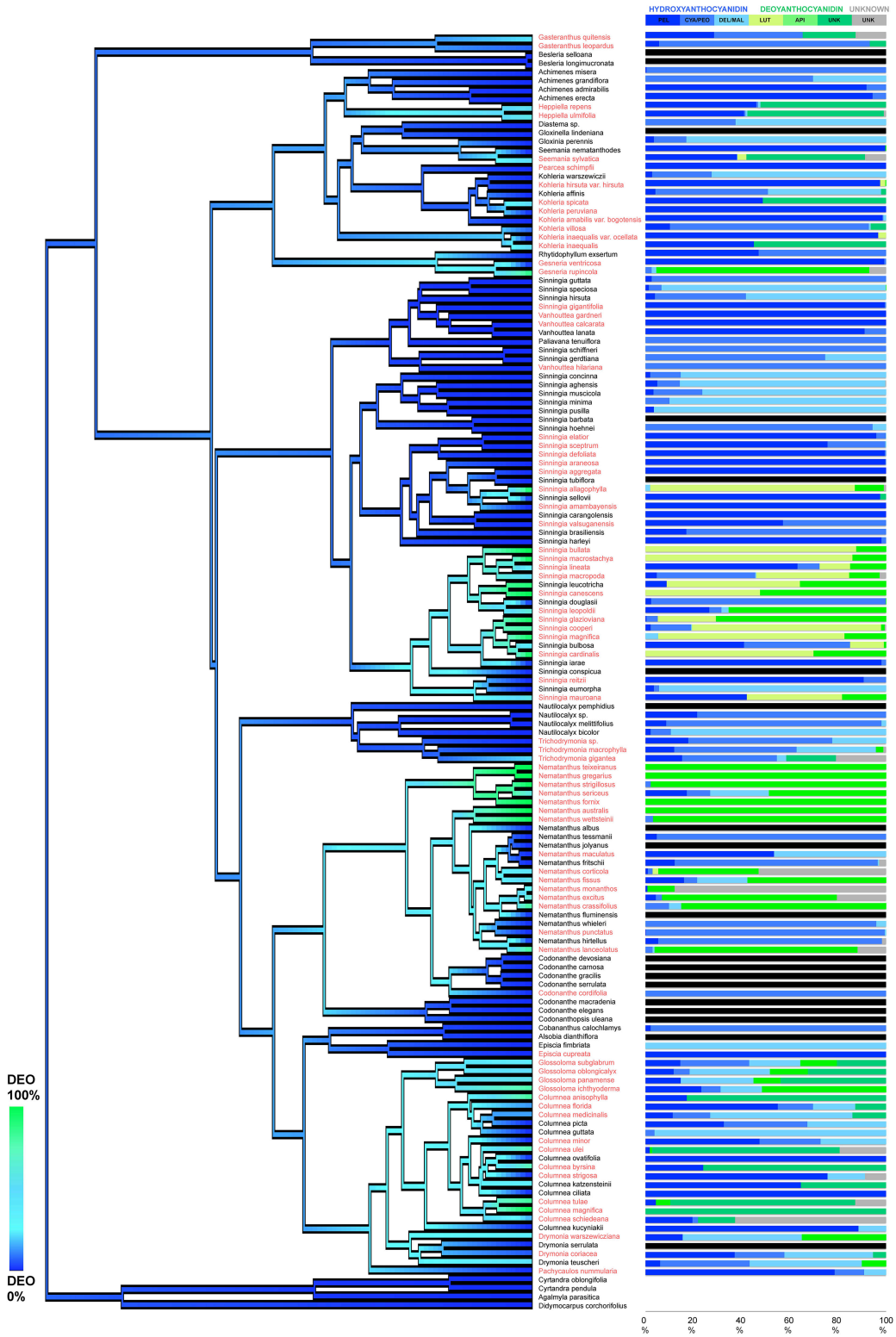


FIGURE 3 | Evolution of deoxyanthocyanin production in the Gesnerioideae. The tree branches are colored based on the reconstructed percentage of the deoxy-anthocyanin presence in the flowers of each species. Species with orange/red flowers are highlighted in red. On the right side of the phylogeny, the percentages of each anthocyanidin are presented in color-coded bars. No-anthocyanin samples are labeled as black bars. PEL, pelargonidin; CYA/PEO, cyanidin and peonidin; DEL/MAL, delphinidin and malvinidin, API, apigenidin, LUT, luteolinidin; UNK (in green): unknown deoxyanthocyanidin; UNK (in gray): unknown anthocyanin. The phylogenetic tree derived from Serrano-Serrano et al. (2017).

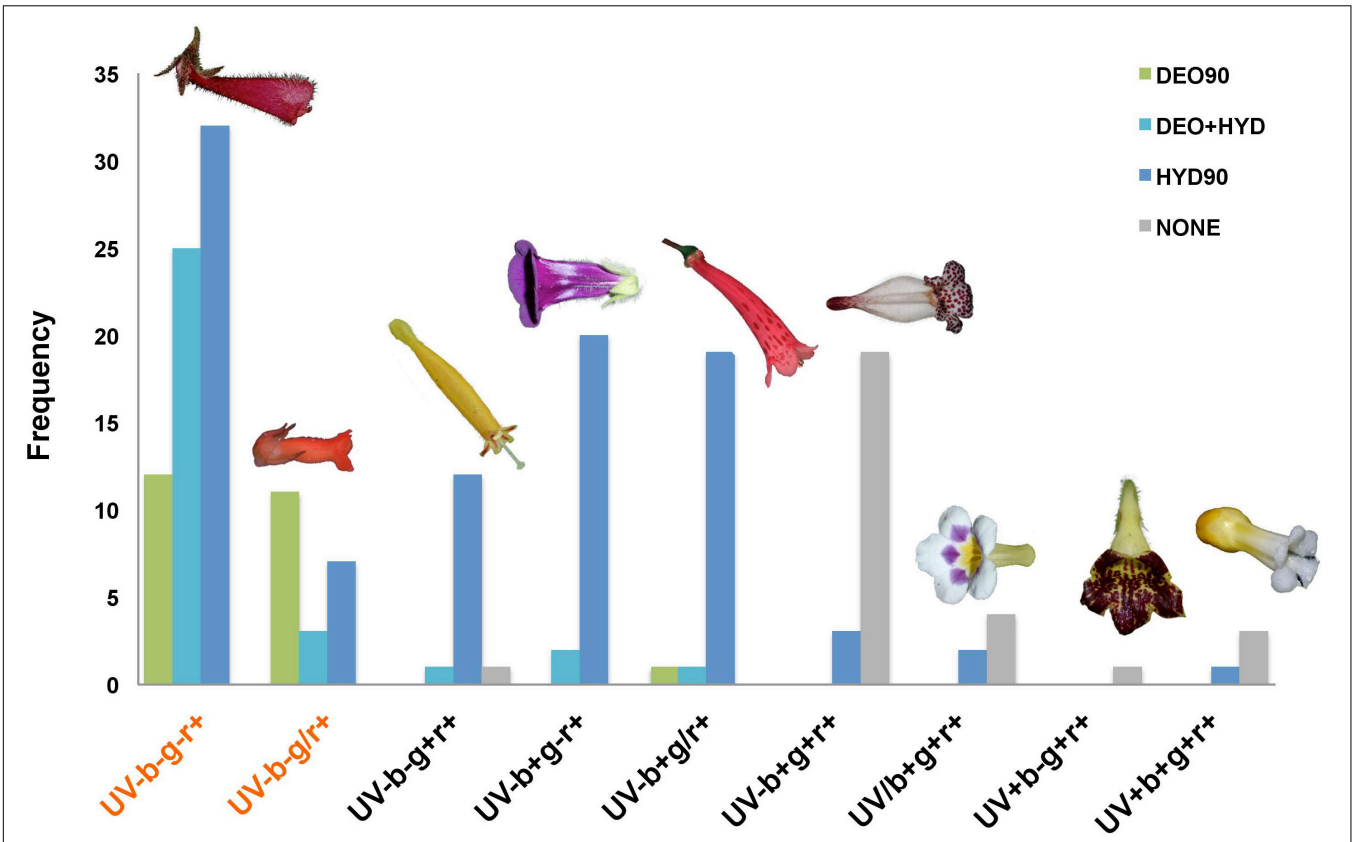


FIGURE 4 | Color groups in the Gesnerioideae flowers. UV: UV (300–400 nm), b: blue (400–500 nm), g: green (500–600 nm), r: red (600–700 nm). –, /, + symbols represent no reflectance, partial reflectance, and full reflectance in the specified region of the spectrum. Color groups representing orange/red hues are shown in red text. DEO90 (green): samples with >90% DEO; DEO+HYD (cyan): samples with both DEO and HYD between 10 and 90%; HYD90 (blue): samples with >90% HYD; NONE: no-anthocyanin samples. Flowers representing each color group are shown at the top of each category. From left to right: *Nematanthus monanthos, Sinningia allagophylla, Columnea purpureamarginata, S. bragae, S. douglasii, N. punctatus, Diastema* sp., *Gasteranthus leopardus,* and *Besleria selloana*.

were significantly lower in HYD-dominant and DEO-dominant samples, respectively (**Table 1** and **Supplementary Table S1**). Lower MAD values indicate better fit for the visual system, meaning that the HYD-dominant samples were better discriminated by bees, whereas DEO-dominant samples were better discriminated by hummingbirds. For minAD values, the most significant differences between the two groups were at the short-wavelength end of the spectrum. When compared to the DEO-dominant samples, HYD-dominant samples had significantly lower minAD400 (short-wavelength receptor for bee vision; $p = 0.0001$) and minAD460 (short-wavelength receptor for hummingbird vision; $p = 0.0001$) values, indicating that HYD-dominant samples were better discriminated by the short-wavelength receptors for both bee and hummingbird visual systems. Lastly, minAD600 values were lower in the DEO-dominant samples than the HYD-dominant samples, which indicates that DEO-dominant samples were better discriminated by the long-wavelength receptors of the hummingbird visual system.

According to the observed pollinator data, out of 52 hummingbird-pollinated flowers, 10 are DEO90, 18 are DEO+HYD, 21 are HYD90, and 3 have no anthocyanins. Out of 16 bee-pollinated flowers, 9 are HYD90, 1 is DEO+HYD, and 6 have no anthocyanins. In other words, all DEO90 and all but one DEO+HYD flowers are exclusively pollinated by hummingbirds (28 samples), whereas HYD90 samples are pollinated by either bees (9 samples) or hummingbirds (21 samples).

DISCUSSION

Changes in floral color have direct impact on the attraction of different pollinator groups, which in turn has a significant effect on angiosperm evolution. However, the biochemical basis of flower color and its impact on flower diversity are only partially understood (Cronk and Ojeda, 2008; Rausher, 2008). Using chemical analysis of anthocyanin pigments and spectral reflectance measurements of 156 species, we found that floral color in the Neotropical Gesneriaceae relies on the biosynthesis of 12 different compounds including not only the common hydroxyanthocyanins but also the rare deoxyanthocyanins that have evolved independently in several lineages. Whereas the hydroxyanthocyanin-producing flowers display a wide range of colors, the deoxyanthocyanin-producing flowers show limited orange/red hues that are discriminated better by hummingbirds

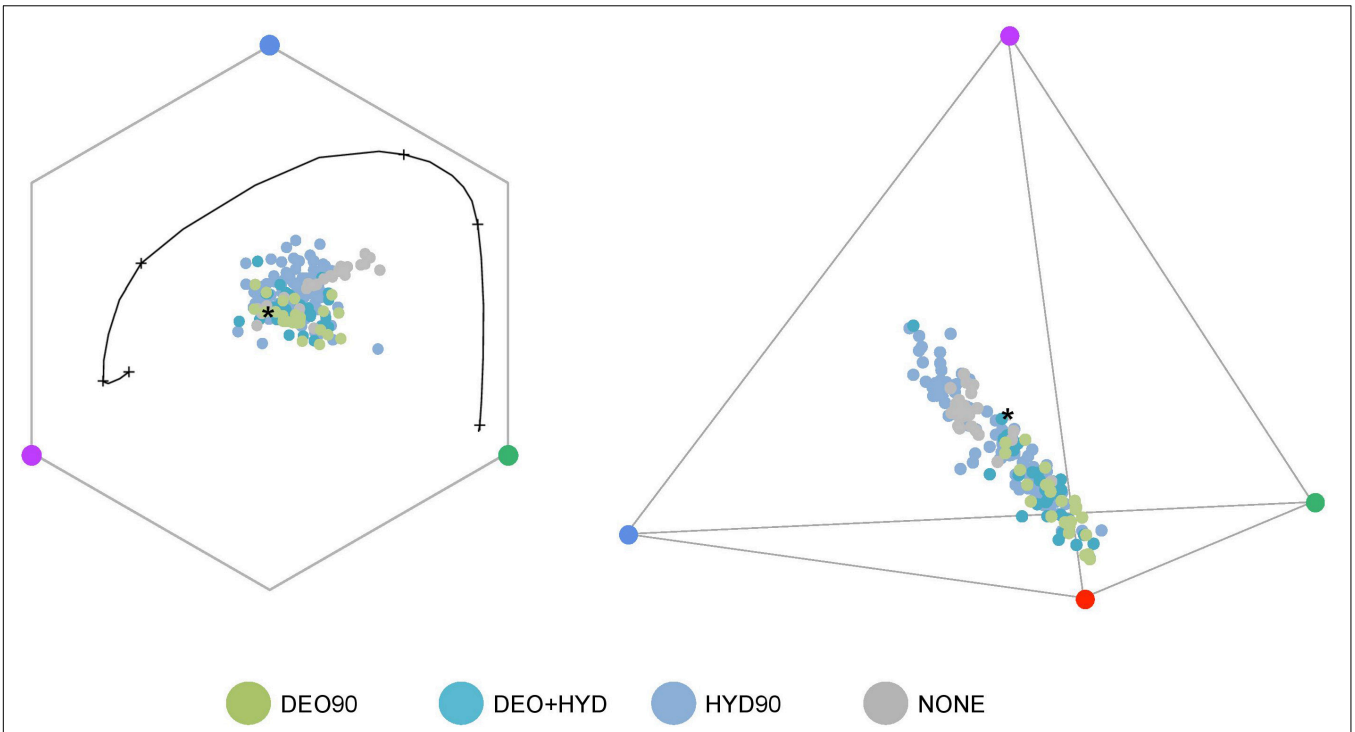


FIGURE 5 | The color loci of Gesnerioideae flowers plotted in bee and hummingbird visual systems. HYD90 (>90% HYD; blue), DEO90 (>90% DEO; green), DEO+HYD (both DEO and HYD between 10 and 90%; cyan) and no-anthocyanin (NONE; gray) samples are shown in the hexagonal color-space of the bee visual system (left) and the tetrahedral color-space of the hummingbird visual system (right). Colored circles in each corner represents a photoreceptor: UV (purple), blue (blue), green (green), and red (red). * represents the achromatic center of the color-space. The black curve on the hexagonal color-space represents the maximum sensitivity of each photoreceptor to a monochromatic light.

than by bees. We also found that the deoxyanthocyanin-producing species were more frequently pollinated by hummingbirds than the hydroxyanthocyanin-producing ones, suggesting that this alternative biochemical pathway may not only constrain the course of floral color evolution but also limit the number of suitable types of pollinators.

The Alternative Branches of the Anthocyanin Pathway in the Gesnerioideae

In most angiosperms that use the ABP, floral color is produced by the common hydroxyanthocyanin derivatives, but the Neotropical Gesneriaceae is one of the few known clades that produces 3-deoxyanthocyanins via an alternative branch of the ABP. We detected these uncommon anthocyanin pigments in more than 30% of the flowers sampled, either alone or in combination with hydroxyanthocyanins. Our result considerably extend the number of Gesnerioideae species with deoxyanthocyanins, which was first documented in the pioneer work of Harborne (1966, 1967) who stressed the potential taxonomical utility of these pigments as a diagnostic feature of the Gesnerioideae subfamily.

To date, deoxyanthocyanins have been mainly recorded in the vegetative parts of disparate groups of seedless plants (mosses and ferns) and angiosperms, where they play a role in defense response against microbial infection and environmental stress (Kawahigashi et al., 2016; Xiong et al., 2019; Piatkowski et al., 2020). Within angiosperms, deoxyanthocyanins have been identified in the leaves of important crops like maize, sorghum and sugarcane (Poaceae), in black tea (*Camellia sinensis,* Theaceae) and in Bignoniaceae (e.g., *Fridericia chica).* Similar to these plant groups, the Gesnerioideae also produce deoxyanthocyanins in their leaves (Harborne, 1967). However, deoxyanthocyanin production in floral tissue and its contribution to pollination signaling is unique to this subfamily.

The frequent presence of both deoxy- and hydroxy-anthocyanins in the same flower indicates that the utility of these two alternative branches of the ABP are not mutually exclusive. We inferred several gains and losses of deoxyanthocyanin production throughout the evolution of the Gesnerioideae, which suggests that the shifts between the two alternative branches of the ABP were frequent and reversible in this subfamily (**Figure 3**). At the molecular level, dihydroflavonol 4-reductase (DFR) and flavanone-3-hydroxylase (F3H) are the key genes for the transition between the hydroxy- and the deoxy-anthocyanin branches of the ABP. In *Sinningia cardinalis*, upregulation of DFR and downregulation of F3H were linked to deoxyanthocyanin production (Winefield et al., 2005). This is achieved by generating flavanone in excess instead of dihydroflavonols, which are subsequently reduced to form the flavan-4-ols precursors rather than the

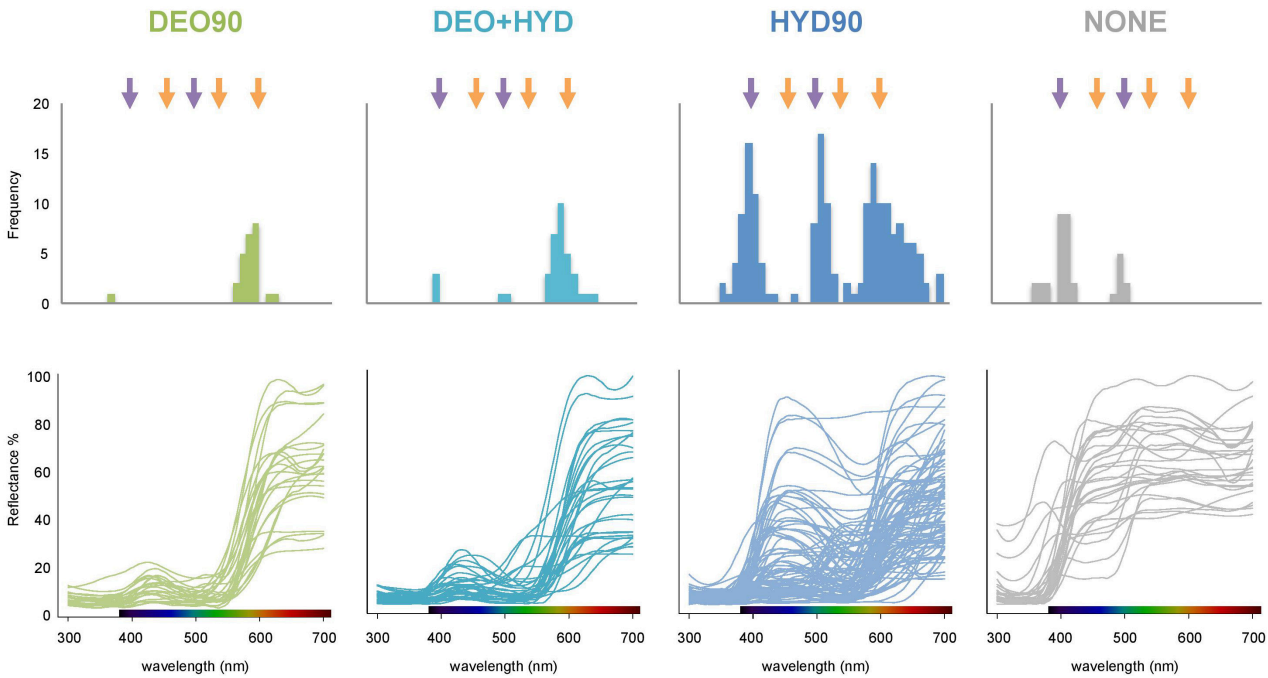


FIGURE 6 | Marker point distribution (top panel) and the reflectance curves (bottom panel) of HYD90 (>90% HYD; blue), DEO90 (>90% DEO; green), DEO+HYD (both DEO and HYD between 10 and 90%; cyan) and no-anthocyanin (NONE; gray) flowers. Purple arrows at 400 and 500 nm represent the hue-discrimination optima for bees, and orange arrows at 460, 540, and 600 nm represent the hue-discrimination optima for hummingbirds. The color panels on the x-axes of the reflectance plots represent the visible light spectrum.

usual flavan-3,4-diol precursors (Stich and Forkmann, 1988a,b). Therefore, the relative proportion of deoxy- and hydroxy-anthocyanins in the Gesnerioideae flowers could be potentially explained by the differences in the regulation of the DFR and F3H, and by different substrate specificity of the DFR copies, which was previously shown in other plant groups (des Marais and Rausher, 2008).

In addition to deoxyanthocyanidins, several hydroxyanthocyanidins (pelargonidin, cyanidins, delphinidin) were also found in various combinations across the Gesnerioideae (**Figure 3** and **Supplementary Figure S1**). A previous study on Solanaceae reported that, even though the species of this family were able to produce all three main groups of anthocyanins, they often produce only one type of anthocyanin or a mix of two anthocyanins with consecutive hydroxylation levels (e.g., pelargonidin-cyanidin or cyanidin-malvidin), and none of the studied species produced all three pigment types (Ng et al., 2018). It has been proposed that the anthocyanin composition is constrained by the stepwise structure of the biosynthetic pathway that leads to the successive production of the red mono-hydroxylated pelargonidin, purple di-hydroxylated cyanidin, and the blue tri-hydroxylated delphinidin pigments (des Marais and Rausher, 2008; Ng et al., 2018). In the Gesnerioideae, anthocyanin composition is less constrained. Although we observed combination of pigments with the same or consecutive hydroxylation levels more often than other combinations (**Supplementary Figure S1**), we also observed pigments with non-consecutive hydroxylation levels together and frequently found up to five pigment types in the same species. This ability to utilize different anthocyanin combinations from two different branches makes anthocyanin-based floral color a highly flexible trait in the Gesnerioideae.

The Effect of Anthocyanin Composition on Floral Color Diversity

Repeated evolution of orange/red flowers occurred frequently in angiosperms (Cronk and Ojeda, 2008; Ng et al., 2015, 2016). Biochemical changes associated with these floral color transitions mainly involve an increased production of red mono-hydroxylated pelargonidin pigments (des Marais and Rausher, 2008), carotenoids (Tanaka et al., 2008), or a combination of both (Ng and Smith, 2016). In Gesnerioideae, orange/red flowers have evolved several times in different lineages, such as in the genera *Columnea* (Schulte et al., 2015), *Drymonia* (Clark et al., 2015), *Nematanthus* (Serrano-Serrano et al., 2015), and *Sinningia* (Perret et al., 2007; **Figure 4**). We found that the convergent evolution of the red color does not rely on the same biochemical basis but on the activation of different branches of the ABP, resulting in the production of up to five different pigments in a single flower. Deoxyanthocyanins and the pelargonidin derivatives contribute to the majority (83%) of the orange/red flowers. Beside these major orange/red pigments, we also found di-hydroxylated cyanidin and tri-hydroxylated malvidin, either alone or in combination with other compounds,

TABLE 1 | Statistical summary of MAD and minAD values for bee and hummingbird visual systems and their comparison between HYD-dominant (>50% HYD) and DEO-dominant (>50% DEO) samples.

		HYD-dominant (n = 111)	DEO-dominant (n = 41)	p-value (phylANOVA)
MAD_Bee	Mean	69.10	83.20	0.001*
	SD	33.78	13.44	
	SEM	3.21	2.10	
MAD_Bird	Mean	29.41	17.07	0.0001*
	SD	17.38	9.84	
	SEM	1.65	1.54	
minAD400	Mean	101.93	176.61	0.0001*
	SD	89.45	38.67	
	SEM	8.49	6.04	
minAD500	Mean	64.35	85.90	0.0981
	SD	43.95	11.24	
	SEM	4.17	1.76	
minAD460	Mean	88.61	122.46	0.0001*
	SD	45.54	15.71	
	SEM	4.32	2.45	
minAD540	Mean	49.87	44.90	0.051
	SD	27.47	11.24	
	SEM	2.61	1.76	
minAD600	Mean	34.14	16.51	0.0029*
	SD	36.71	8.97	
	SEM	3.48	1.40	

*Lower MAD and minAD values indicate higher discrimination abilities. *Indicates statistical significance. SD, standard deviation; SEM, Standard error of the mean.*

in some other orange/red flowers like in *Vanhouttea hilariana*. Although these pigments are frequently associated with pink and purple/blue flowers, it has been shown that these anthocyanins can produce red under low vacuolar pH conditions (Yoshida et al., 2003; Tanaka et al., 2008). These results demonstrate that Gesnerioideae evolved several different biochemical solutions to produce orange/red flowers, which indicates that convergent evolution of orange/red color does not result from parallel changes at the biochemical level but instead produced via the activation of different branches of the ABP.

Beside anthocyanins, other pigment types may also be involved in the floral color in the Gesnerioideae. For example, the pale green flowers of *Rhytidophyllum exsertum* and *Sinningia brasiliensis* have chloroplast in their parenchyma, and the overlay of cyanidin-producing epidermal cells result in the brown dots typical of these bat-pollinated flowers (Sanmartin-Gajardo and Sazima, 2005; Martén-Rodríguez et al., 2015). Carotenoids may also contribute to the yellow floral colors in the Gesnerioideae samples where no anthocyanidins were detected. To date, carotenoids have been recorded in the genus *Achimenes*, *Columnea* (*C. crassifolia, C. linearis, C. rutilans,* and *C. woodii*) and in *Titanotrichum oldhamii* (Harborne, 1967; Roberts and Roalson, 2017), but the overall presence of carotenoids in the Gesneriaceae family has not been studied in detail.

The Effect of Floral Anthocyanin Type on Color Discrimination of Pollinators

Flower color has a direct impact on their visual discrimination by pollinators. A fit between visual systems and specific color signals has been observed in communities with bees and birds as pollinators (Burd et al., 2014; Papiorek et al., 2016; Shrestha et al., 2016; Camargo et al., 2019). However, little is known about how these color signals evolved to match the visual systems of their principal pollinators during plant radiations. Using both analysis of spectral marker points (**Figure 6**) and color space models for bees and birds (**Figure 5**), we show that the extent of floral color diversity in Gesnerioideae depends on which ABP branch is activated. Species relying on the hydroxyanthocyanin branch of the ABP pathway display a wide range of color and marker points, and they match the visual capacities of both bees and hummingbirds. The visual color space occupied by deoxyanthocyanin-producing flowers is smaller than the hydroxyanthocyanin-producing flowers, and these flower colors better match the visual capacities of hummingbirds. The effect of these floral spectral specificity on pollinator preferences is confirmed by pollinator observation data, which indicate that hydroxyanthocyanin-producing species are either pollinated by bees or hummingbirds, whereas flowers with deoxyanthocyanin are only pollinated by hummingbirds. The lack of bee-pollination within the deoxyanthocyanin-producing flowers indicate that this type of floral pigment is particularly efficient at limiting bee visitation by constraining flower reflectance in the long-wavelength ranges that are better detected by hummingbirds than bees (Lunau et al., 2011; Bergamo et al., 2016).

Even though the shift from the hydroxy- to deoxy-anthocyanin branch may decrease the subsequent phenotypic and ecological diversification of a clade, relying on deoxyanthocyanins as floral pigments might still be evolutionary beneficial. Indeed, constraining the deoxyanthocyanin-producing clades to rely only on hummingbird pollination maybe advantageous, because this pollinator group is particularly efficient in the rainforests of mountain systems with a high Gesnerioideae diversity (Perret et al., 2013; González et al., 2015). In addition, transitions to hummingbird pollination has been shown to promote diversification not only in the Gesnerioideae (Roalson and Roberts, 2016; Serrano-Serrano et al., 2017), but also in many other Neotropical clades (Kay et al., 2005; Wilson et al., 2006; Schmidt-Lebuhn et al., 2007; Tripp and Manos, 2008).

CONCLUSION

We have shown that the Gesnerioideae flowers show remarkable color diversity, which is due to the use of the ABP up to its full capacity, including the deoxyanthocyanin branch, which is rarely employed in angiosperms. Utilizing both branches in the ABP provides Gesnerioideae with alternative options for floral color production without forcing an evolutionary dead-end, resulting in extraordinary floral color diversity and different signaling strategies adapted for bees and hummingbirds. By improving our understanding the biochemical basis of floral color in the Gesnerioideae, we provide clear perspectives to identify the genetic changes responsible for floral color shifts and

test the effects of these transitions on plant-pollinator interactions and angiosperm diversification.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EO contributed to data collection, data analysis, and manuscript writing. KD contributed to data collection and data analysis. MW, LC, and CG contributed to data collection and manuscript review. GG contributed to data analysis and manuscript review. MP contributed to supervision and manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.604389/full#supplementary-material

REFERENCES

Albert, N. W., Davies, K. M., Lewis, D. H., Zhang, H., Montefiori, M., Brendolise, C., et al. (2014). A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *Plant Cell* 26, 962–980. doi: 10.1105/tpc.113.122069

Bergamo, P. J., Rech, A. R., Brito, V. L., and Sazima, M. (2016). Flower colour and visitation rates of Costus arabicus support the 'bee avoidance' hypothesis for red-reflecting hummingbird-pollinated flowers. *Funct. Ecol.* 30, 710–720. doi: 10.1111/1365-2435.12537

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B Biol. Sci.* 281:20132862. doi: 10.1098/rspb.2013.2862

Calenge, C. (2006). The package "adehabitat" for the R software: a tool for the analysis of space and habitat use by animals. *Ecol. Model.* 197, 516–519. doi: 10.1016/j.ecolmodel.2006.03.017

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543.

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181.

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vis. Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Clark, J. L., Clavijo, L., and Muchhala, N. (2015). Convergence of anti-bee pollination mechanisms in the Neotropical plant genus Drymonia (Gesneriaceae). *Evol. Ecol.* 29, 355–377. doi: 10.1007/s10682-014-9729-4

Cronk, Q., and Ojeda, I. (2008). Bird-pollinated flowers in an evolutionary and molecular context. *J. Exp. Bot.* 59, 715–727. doi: 10.1093/jxb/ern009

des Marais, D. L., and Rausher, M. D. (2008). Escape from adaptive conflict after duplication in an anthocyanin pathway gene. *Nature* 454, 762–765. doi: 10.1038/nature07092

Dray, S., and Dufour, A. B. (2007). The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* 22, 1–20.

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G., Simonov, V., and Wong, B. B. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Emmerton, J., and Delius, J. D. (1980). Wavelength discrimination in the 'visible' and ultraviolet spectrum by pigeons. *J. Comp. Physiol.* 141, 47–52. doi: 10.1007/bf00611877

González, A. M., Dalsgaard, B., Nogués-Bravo, D., Graham, C. H., Schleuning, M., Maruyama, P. K., et al. (2015). The macroecology of phylogenetically structured hummingbird–plant networks. *Glob. Ecol. Biogeogr.* 24, 1212–1224. doi: 10.1111/geb.12355

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Harborne, J. B. (1966). Comparative biochemistry of flavonoids-II.: 3-Desoxyanthocyanins and their systematic distribution in ferns and gesnerads. *Phytochemistry* 5, 589–600. doi: 10.1016/s0031-9422(00)83637-7

Harborne, J. B. (1967). Comparative biochemistry of the flavonoids-VI.: flavonoid patterns in the Bignoniaceae and the Gesneriaceae. *Phytochemistry* 6, 1643–1651. doi: 10.1016/s0031-9422(00)82897-6

Herrera, G., Zagal, J. C., Diaz, M., Fernández, M. J., Vielma, A., Cure, M., et al. (2008). Spectral sensitivities of photoreceptors and their role in colour discrimination in the green-backed firecrown hummingbird (Sephanoides sephaniodes). *J. Comp. Physiol. A* 194:785. doi: 10.1007/s00359-008-0349-8

Hoballah, M. E., Gübitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., et al. (2007). Single gene–mediated shift in pollinator attraction in Petunia. *Plant Cell* 19, 779–790. doi: 10.1105/tpc.106.048694

Holton, T. A., and Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7:1071. doi: 10.1105/tpc.7.7.1071

Kassambara, A., and Mundt, F. (2020). *Factoextra: Extract and Visualize the Results of Multivariate Data Analyses*. Available online at: https://CRAN.R-project.org/package=factoextra (accessed August 12, 2020).

Kawahigashi, H., Kasuga, S., Sawada, Y., Yonemaru, J. I., Ando, T., Kanamori, H., et al. (2016). The sorghum gene for leaf color changes upon wounding (P) encodes a flavanone 4-reductase in the 3-deoxyanthocyanidin biosynthesis pathway. *G3* 6, 1439–1447. doi: 10.1534/g3.115.026104

Kay, K. M., Reeves, P. A., Olmstead, R. G., and Schemske, D. W. (2005). Rapid speciation and the evolution of hummingbird pollination in neotropical Costus subgenus Costus (Costaceae): evidence from nrDNA ITS and ETS sequences. *Am. J. Bot.* 92, 1899–1910. doi: 10.3732/ajb.92.11.1899

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Maddison, W. P., and Maddison, D. R. (2014). *Mesquite: A Modular System for Evolutionary Analysis. Version 3*.

Maia, R., Eliason, C. M., Bitton, P. P., Doucet, S. M., and Shawkey, M. D. (2013). pavo: an R package for the analysis, visualization and organization of spectral data. *Methods Ecol. Evol.* 4, 906–913.

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210x.13174

Martén-Rodríguez, S., Quesada, M., Castro, A. A., Lopezaraiza-Mikel, M., and Fenster, C. B. (2015). A comparison of reproductive strategies between island and mainland Caribbean Gesneriaceae. *J. Ecol.* 103, 1190–1204. doi: 10.1111/1365-2745.12457

Ng, J., Freitas, L. B., and Smith, S. D. (2018). Stepwise evolution of floral pigmentation predicted by biochemical pathway structure. *Evolution* 72, 2792–2802. doi: 10.1111/evo.13589

Ng, J., and Smith, S. D. (2016). Widespread flower color convergence in Solanaceae via alternate biochemical pathways. *New Phytol.* 209, 407–417. doi: 10.1111/nph.13576

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator−dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Perret, M., Chautems, A., De Araujo, A. O., and Salamin, N. (2013). Temporal and spatial origin of Gesneriaceae in the New World inferred from plastid DNA sequences. *Bot. J. Linnean Soc.* 171, 61–79. doi: 10.1111/j.1095-8339.2012.01303.x

Perret, M., Chautems, A., Spichiger, R., Barraclough, T. G., and Savolainen, V. (2007). The geographical pattern of speciation and floral diversification in the Neotropics: the tribe Sinningieae (Gesneriaceae) as a case study. *Evolution* 61, 1641–1660. doi: 10.1111/j.1558-5646.2007.00136.x

Piatkowski, B. T., Imwattana, K., Tripp, E. A., Weston, D. J., Healey, A., Schmutz, J., et al. (2020). Phylogenomics reveals convergent evolution of red-violet coloration in land plants and the origins of the anthocyanin biosynthetic pathway. *Mol. Phylogenet. Evol.* 151:106904. doi: 10.1016/j.ympev.2020.106904

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210x.2011.00169.x

Roalson, E. H., and Roberts, W. R. (2016). Distinct processes drive diversification in different clades of Gesneriaceae. *Syst. Biol.* 65, 662–684. doi: 10.1093/sysbio/syw012

Roberts, W. R., and Roalson, E. H. (2017). Comparative transcriptome analyses of flower development in four species of Achimenes (Gesneriaceae). *BMC Genom.* 18:240. doi: 10.1186/s12864-017-3623-8

Roberts, W. R., and Roalson, E. H. (2020). Co-expression clustering across flower development identifies modules for diverse floral forms in Achimenes (Gesneriaceae). *PeerJ* 8:e8778. doi: 10.7717/peerj.8778

Sanmartin-Gajardo, I., and Sazima, M. (2005). Chiropterophily in Sinningieae (Gesneriaceae): Sinningia brasiliensis and Paliavana prasinata are bat-pollinated, but P. sericiflora is not. Not yet? *Ann. Bot.* 95, 1097–1103. doi: 10.1093/aob/mci124

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Schmidt-Lebuhn, A. N., Kessler, M., and Hensen, I. (2007). Hummingbirds as drivers of plant speciation? *Trends Plant Sci.* 12, 329–331. doi: 10.1016/j.tplants.2007.06.009

Schulte, L. J., Clark, J. L., Novak, S. J., Jeffries, S. K., and Smith, J. F. (2015). Speciation within Columnea section angustiflora (Gesneriaceae): islands, pollinators and climate. *Mol. Phylogenet. Evol.* 84, 125–144. doi: 10.1016/j.ympev.2014.12.008

Serrano-Serrano, M. L., Perret, M., Guignard, M., Chautems, A., Silvestro, D., and Salamin, N. (2015). Decoupled evolution of floral traits and climatic preferences in a clade of Neotropical Gesneriaceae. *BMC Evol. Biol.* 15:247. doi: 10.1186/s12862-015-0527-6

Serrano-Serrano, M. L., Rolland, J., Clark, J. L., Salamin, N., and Perret, M. (2017). Hummingbird pollination and the diversification of angiosperms: an old and successful association in Gesneriaceae. *Proc. R. Soc. B Biol. Sci.* 284:20162816. doi: 10.1098/rspb.2016.2816

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Sobel, J. M., and Streisfeld, M. A. (2013). Flower color as a model system for studies of plant evo-devo. *Front. Plant Sci.* 4:321. doi: 10.3389/fpls.2013.00321

Stich, K., and Forkmann, G. (1988a). Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*. *Phytochemistry* 27, 785–789. doi: 10.1016/0031-9422(88)84093-7

Stich, K., and Forkmann, G. (1988b). Studies on columnidin biosynthesis with flower extracts from *Columnea hybrida*. *Zeitschrift für Naturforschung C* 43, 311–314. doi: 10.1515/znc-1988-3-426

Stoddard, M. C., Eyster, H. N., Hogan, B. G., Morris, D. H., Soucy, E. R., and Inouye, D. W. (2020). Wild hummingbirds discriminate nonspectral colors. *Proc. Natl. Acad. Sci. U.S.A.* 117, 15112–15122. doi: 10.1073/pnas.1919377117

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313x.2008.03447.x

Tripp, E. A., and Manos, P. S. (2008). Is floral specialization an evolutionary dead-end? Pollination system transitions in Ruellia (Acanthaceae). *Evolution* 62, 1712–1737. doi: 10.1111/j.1558-5646.2008.00398.x

von Helversen, O. (1972). Zur spektralen Unterschiedsempfindlichkeit der honigbiene. *J. Comp. Physiol.* 80, 439–472. doi: 10.1007/bf00696438

Warnes, G. R. (2005). *gmodels: Greg's Miscellaneous Functions., 2005. R package version, 2(6), 603*.

Wheeler, L. C., and Smith, S. D. (2019). Computational modeling of anthocyanin pathway evolution: Biases, hotspots, and trade-offs. *Integrative and comparative biology* 59, 585–598. doi: 10.1093/icb/icz049

Wilson, P., Castellanos, M. C., Wolfe, A. D., and Thomson, J. D. (2006). "Shifts between bee and bird pollination in Penstemons," in *Plant-Pollinator Interactions: From Specialization To Generalization*, eds N. M. Waser and J. Ollerton (Chicago, CA: The University of Chicago Press), 47–68.

Winefield, C. S., Lewis, D. H., Swinny, E. E., Zhang, H., Arathoon, H. S., Fischer, T. C., et al. (2005). Investigation of the biosynthesis of 3-deoxyanthocyanins in *Sinningia cardinalis*. *Physiol. Plant.* 124, 419–430. doi: 10.1111/j.1399-3054.2005.00531.x

Xiong, Y., Zhang, P., Warner, R. D., and Fang, Z. (2019). 3-Deoxyanthocyanidin colorant: nature, health, synthesis, and food applications. *Comp. Rev. Food Sci. Food Safety* 18, 1533–1549. doi: 10.1111/1541-4337.12476

Yoshida, K., Toyama-Kato, Y., Kameda, K., and Kondo, T. (2003). Sepal color variation of Hydrangea macrophylla and vacuolar pH measured with a proton-selective microelectrode. *Plant Cell Physiol.* 44, 262–268. doi: 10.1093/pcp/pcg033

Zhao, D., and Tao, J. (2015). Recent advances on the development and regulation of flower color in ornamental plants. *Front. Plant Sci.* 6:261. doi: 10.3389/fpls.2015.00261

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Floral Color Diversity: How Are Signals Shaped by Elevational Gradient on the Tropical–Subtropical Mountainous Island of Taiwan?

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Pollinators with different vision are a key driver of flower coloration. Islands provide important insights into evolutionary processes, and previous work suggests islands may have restricted flower colors. Due to both species richness with high endemism in tropical–subtropical environments, and potentially changing pollinator distributions with altitude, we evaluated flower color diversity across the mountainous island of Taiwan in a comparative framework to understand the cause of color diversity. We sampled flower color signaling on the tropical–subtropical island of Taiwan considering altitudes from sea level to 3300 m to inform how over-dispersion, random processes or clustering may influence flower signaling. We employed a model of bee color space to plot loci from 727 species to enable direct comparisons to data sets from continental studies representing Northern and Southern Hemispheres, and also a continental mountain region. We observed that flower color diversity was similar to flowers that exist in mainland continental studies, and also showed evidence that flowers predominantly had evolved color signals that closely matched bee color preferences. At high altitudes floras tend to be phylogenetically clustered rather than over-dispersed, and their floral colors exhibited weak phylogenetic signal which is consistent with character displacement that facilitated the co-existence of related species. Overall flower color signaling on a tropical–subtropical island is mainly influenced by color preferences of key bee pollinators, a pattern consistent with continental studies.

Keywords: flowers, bee vision, phylogeny, community, altitude, tropical–subtropical, island

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INTRODUCTION

Floral color is a key functional trait that affects plant–pollinator interactions (Oberrath and Böhning Gaese, 1999; Bradshaw and Schemske, 2003; Ômura and Honda, 2005), contributing to biodiversity and structure of plant communities (Sargent and Ackerly, 2008; Schemske et al., 2009). The variation of floral color diversity (FCD) may reflect how plant–pollinator interactions and evolutionary history shape color signaling (Weevers, 1952; Chittka and Menzel, 1992; McEwen and Vamosi, 2010). Whilst there has been increased interest on the studies of FCD for continental floras

around the world (Arnold et al., 2009a,b; Dyer et al., 2012; Shrestha et al., 2014; Gray et al., 2018), relevant studies of island floras are rarely reported (Makino and Yokoyama, 2015; Shrestha et al., 2016).

Island data can provide important insights for a better understanding the global patterns of floral color resulting from plant–pollinator interactions, and how threats like species invasion, habitats loss and/or pollinator extinction (Cox and Elmqvist, 2000; Brooks et al., 2002; van Kleunen et al., 2015) may impact environments. Compared to continental environments, islands can provide an assessable model with insular nature to study the FCD (Emerson and Gillespie, 2008). Interestingly, previous studies report that oceanic island floras [e.g., Macquarie Island (MI) (Shrestha et al., 2016), Juan Fernández Islands (JFI) (Bernardello et al., 2001) and sub-Antarctic Campbell Island (Lord et al., 2013) display simple and inconspicuous colors and have low FCD. Even on larger oceanic islands, floras in New Zealand (NZ) mountains consist of constrained flower color signals, exhibiting much lower FCD (Bischoff et al., 2013b) when compared to its adjacent continental island of Australia (Webb and Kelly, 1993; Dyer et al., 2012). It's worth noting that the low species richness of these oceanic islands, e.g., MI (37 angiosperm species, de Salas and Baker (2015) and JFI (152 species), may more or less determine their low FCD, as plant communities with larger species richness would often, but not always, have greater FCD (Arnold et al., 2009b; Shrestha et al., 2014; Bergamo et al., 2018; Gray et al., 2018).

Oceanic island plants were mainly colonized from continents within a short evolutionary history and often lacks guilds of pollinators, thus low FCD was expected. Unlike oceanic islands, Taiwan is an atypical island, i.e., isolation with partial reconnection during glaciation and it has been repeatedly in contact with the Asian continent (Voris, 2000), which allows plants to readily migrate into Taiwan (Hsieh, 2002; Huang, 2011; Li et al., 2013). Moreover, Taiwan is situated on the transition zone between subtropical and tropical region, potentially contributing to a greater FCD, as biota have long been thought to exhibit greater color diversity in tropics than high latitudes (Wilson and von Neumann, 1972; Adams et al., 2014). The islands of Taiwan are located at the boundary between the Holarctic and Paleotropical floristic kingdoms indicating the diverse evolutionary origins of local flora (Hsieh, 2002). Indeed, Taiwan harbors greater species richness (up to 3,500 flowering species) and higher specific endemism (26.1% of native plants) than other oceanic islands (Hsieh, 2002), we thus might expect a greater FCD in Taiwan, especially with respect to other island studies.

In addition to having high species richness, Taiwan also has mountainous topography, with steep altitudinal gradients up to 3952 m a.s.l. and accessible flowering plants to 3300 m a.s.l. within a relatively short distance. This creates altitudinal vegetation zones from tropical/foothill lowland evergreen broad-leaved forest to subtropical evergreen and temperature deciduous mountain forest, and to mountain peaks as cool temperate upper-montane coniferous and subalpine boreal forest or alpine tundra (Li et al., 2013). The percentage of flowering plant endemism in Taiwan therefore increases dramatically along altitude from 15% at sea level to reach 60% among most mountain peaks (Hsieh, 2002). This provides us with an appealing opportunity to incorporate how floral colors are shaped along altitudinal gradients of the tropical–subtropical island, as previous works have studied the variation of FCD along altitudes in several continents (Arnold et al., 2009b; Shrestha et al., 2014; Gray et al., 2018), but to the best of our knowledge this has not been tested on an island.

Considering mountainous environments, Bergamo *et al.* analyzed 71 plant species in 700–1800 m a.s.l. and found the FCD was greater in low altitudes than that in middle and high altitudes (Bergamo et al., 2018), whereas Shrestha et al. (2014) analyzed 107 plant species in Nepal and found a converse pattern, i.e., greater the FCD in subalpine region (3000–4100 m, $n = 61$) than subtropical regions (900–2000 m, $n = 46$). Interestingly, in the Himalayan mountains of Nepal there is also a high diversity of bumblebees and some other insects (Thapa, 2000; Williams et al., 2010) that act as pollinators of flowering plants (Shrestha et al., 2014; Paudel et al., 2015, 2016, 2018). These studies suggested that the species richness (represented by sample sizes) may be a main factor influencing FCD, and altitude as a factor may only mediate probability of species richness depending upon temperature range in a particular region of the world. To contribute to our understanding of the complexity of FCD and climatic conditions due to changing altitude, our work included a large sample size in each altitude group (>140 species) and covered an entire altitude gradient over which flowering plants exist in Taiwan (from 0 to 3300 m). This enables formally testing if species richness is a key factor to determine the FCD in an environment.

In addition, floral color assembly (FCA) demonstrates the similarity of floral colors between species within a given region, i.e., if sympatric floras tend to display similar colors (cluster) or divergent colors (over-dispersion). When compared to FCD, an investigation of FCA can enable interpretations of how species evolutionary history and plant–pollinator interaction shape the floral colors within different plant communities (Gumbert et al., 1999; McEwen and Vamosi, 2010; Makino and Yokoyama, 2015).

Several studies used null models based on random assembly to evaluate the pattern of FCA, and revealed that FCA was non-random in most plant communities and highly shaped by the plant–pollinator interaction (de Jager et al., 2011; Muchhala et al., 2014; Makino and Yokoyama, 2015; Ohashi et al., 2015; Kemp et al., 2019), although evidence of random assembly also exists for the continental island of Australia (Shrestha et al., 2019b). Competition for pollinator visitations was the major selection force to shape the FCA of plant communities in high latitudes and high altitudes, which often caused floral color over-dispersion (Muchhala et al., 2014; van der Kooi et al., 2016; Bergamo et al., 2018), but there is also some evidence of clustering at high altitudes (Bergamo et al., 2018). As both number and abundance of pollinators are thought to generally decrease with increasing altitudes (Warren et al., 1988; Bingham and Orthner, 1998), but see Shrestha et al. (2014) for a different effect in the Himalayan mountains, it is plausible this may affect FCA.

At a global scale large islands have relatively more bee species (ca. 0.0011–0.0420 spp. per square kilometer, and Taiwan 0.005)

than are present on continental land masses (ca. 0.0004–0.0015) (Buchmann, 1994). Bees are often more effective in pollination than other insects, e.g., bees deliver more pollen in each visitation, and visit more flowers per minute than the flies (Bingham and Orthner, 1998; Bischoff et al., 2013a) as well as often exhibiting flower constancy (Chittka et al., 1999). In our current study, we thus not only investigated the floral colors in the human color category, but also translated their reflectance spectra into color loci in sensory color space of the bee (bee hexagon) which permits a robust interpretation of how important bee pollinators perceive floral color signals (Chittka, 1992; Dyer, 2006; Kemp et al., 2019; Shrestha et al., 2019b). This advanced technique is possible because for bees there is detailed knowledge about their phylogenetically conserved trichromatic vision including receptors, neural processing and calibration with behavioral data (Chittka, 1992; Dyer et al., 2011; Garcia et al., 2017; Shrestha et al., 2019a,b). The bee color hexagon model was used to quantify the FCD (Shrestha et al., 2014, 2016), and was also used to evaluate the FCA by applying the mean pair-wise distance (MPD) of the color loci (McEwen and Vamosi, 2010; Makino and Yokoyama, 2015; Shrestha et al., 2019b).

Studies of floras from both Southern and Northern Hemispheres consistently exhibited a predominant direction of color loci in bee hexagon (Chittka et al., 1994; Dyer et al., 2012; Shrestha et al., 2014, 2019b), which overlapped with the short-wavelength "blue" color preferences of several bee species that have been formally tested (Giurfa et al., 1995; Raine and Chittka, 2005; Dyer et al., 2016). Such distinct color preferences likely exist due to the overlap of the phylogenetically conserved color photoreceptors in bees from around the world (Peitsch et al., 1992; Giurfa et al., 1995; Raine and Chittka, 2005; Morawetz et al., 2013; Dyer et al., 2016), and strongly suggest that the bee color preferences are the major selection force that shape the floral colors in regions where bees are common pollinators (Shrestha et al., 2019b). Indeed, when bees are not the pollinator of flowers then very different distributions of flower colors are observed (Lunau et al., 2011; Shrestha et al., 2013, 2016, 2019a; Burd et al., 2014; Camargo et al., 2019).

The multiple origins of Taiwan flora and complex island habitats thus may generate unique floral colors as flowers adapt to different pollinators along elevation gradients. We therefore would like to test whether flower color diversity is comparable to these adjacent continent and islands. We also would like to ask whether flower color is constrained for attracting limited pollinators on islands or diverges for competing pollinator visiting. Our null position is that flower color in Taiwan is not significantly different to mainland continental studies. Thus, in the current study, we firstly evaluate whether the floras in tropical–subtropical island Taiwan exhibited low FCD consistent with other island studies, or if the favorable conditions of a tropically located mountain island may promote higher FCD consistent with, or even greater than, mainland continental studies. We secondly investigate if there is evidence of variation of FCD considering three defined different altitudinal zones in TW, and evaluate if any observed variation might be dependent upon species richness. For our analyses we consider the evidence or whether FCA of plants in TW are over-dispersed, random or clustered based on low, mid and high-altitude plants using the phylogenetic-informed analysis, whereby we could interpret the possible driving force and ecological process accounting for the patterns of FCA. Finally, we use a comparative framework to evaluate if bee color preferences are also a likely factor influencing the distribution of floral colors in Taiwan, as has been recently demonstrated in other parts of the world.

MATERIALS AND METHODS

Study Area and Sample Collection

The main island of Taiwan lies in East Asia located in between 21.916107 N - 25.086991 N, 120.787456 E - 121.899446 E with a land area of 35,808 square kilometers. The island is surrounded by South and East China Seas, and at the nearest point is 130 km from any other major continental land mass (**Figure 1**). The main island lies in a complex tectonic region of Yangtze Plate to the west and north, the Okinawa Plate on the north-east, and the Philippine Mobile Belt on the east and south, formed approximately four to five million years ago (Wu, 1978; Biq et al., 1985). Moreover, many high mountains in Taiwan (over 100 peaks over 3000 m a.s.l.) have created topographically isolated habitats and fast-changing climatic zones (from tropical low land forest to alpine tundra) along elevation changes. The multiple origins of Taiwan flora and complex island habitats thus may have potentially generated very unique FCD in different habitats along elevation. However, very little was known about Taiwanese or even Asian flower coloration with respect to animal pollinator color vision prior to our study.

We selected our study sites in the Taiwanese main island and two nearby offshore islands named Green Island and Orchid Island. We classified these sites into different altitudinal regions, i.e., low (0–900 m a.s.l.), middle (1500–2200 m a.s.l.) and high-altitude regions (2800–3300 m a.s.l.) (**Supplementary Table S1**). Sample sites in low-altitude comprise multiple types of vegetation zones including coastal, tropical and subtropical mountain zonal forest; middle sites are dominated by subtropical evergreen cloud forest, while the high-altitude sample site contained high-mountain coniferous woodlands and forests (Li et al., 2013). We chose a total of 18 sample sites from three altitudinal regions: 11 sites in low, 6 sites in middle, and 1 site in high-altitude (details in **Supplementary Table S1**). These sampling sites cover major altitudinal vegetation zones in Taiwan. We mostly choose the National Parks in different altitudinal regions, which were well-studied and represent all vegetation types of the study region. The number of sites tested reflected availability in respective National Parks. We sampled one high-altitudinal site covering a 10 km^2 mountain range of several higher than 3200 m mountain peaks at the Taroko national park. All flower samples were native to Taiwan and were collected from March 2016 to September 2017, covering two periods of the peak blooming (Tai, 2018). Our dataset comprised of a total of 727 native flowering species (399 species in low-altitude, 186 species in middle-altitude, and 142 species in high-altitude) [see details **Supplementary Table S2**: species list and altitude, *Dryad digital*

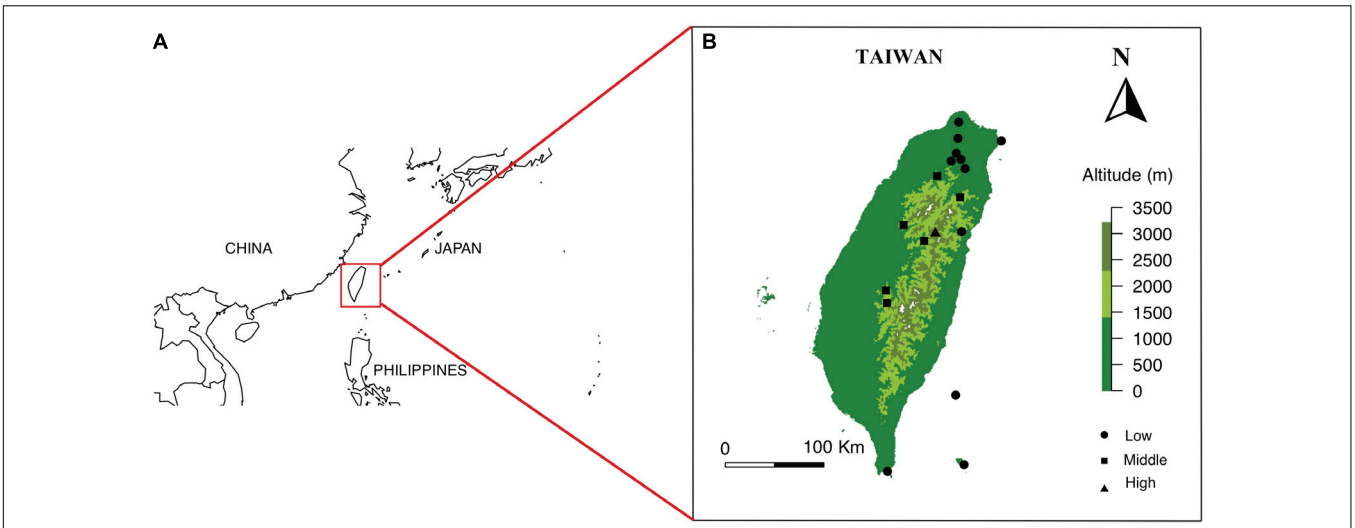


FIGURE 1 | Location of Taiwan in red square **(A)**, and the maps of 18 sampling sites from three altitudinal regions in Taiwan **(B)**. Maps are prepared using package Maps in R version 3.4.4.

repository (Tai et al., 2020)] including ca 33% endemic species to Taiwan. Some example flowers collected at different altitudinal regions is given in **Figure 2**. We also used the data from Australia (Burd et al., 2014), Nepal (Shrestha et al., 2014), and Japan (Makino and Yokoyama, 2015) to enable a comparative framework to other regions.

Floral Color Measurement

Floral colors were measured using an Ocean Optics spectrophotometer (Ocean Optics Inc., USB-4000+, United States) with a UV-VIS-NIR light source (Ocean Optics Inc., DH-2000-BAL, United States) and a quartz fiber-optic probe (Ocean Optics Inc., Lab-grade Reflection Probes, United States). The reflectance spectra were measured from 300 to 700 nm (see **Supplementary Method S1**) and then processed by the software OCEAN VIEW (Ocean Optics Inc., United States). Additional details for the measurement of reflectance spectra were given in **Supplementary Method S1** and **Supplementary Figure S1** for the details of marker point comparison to show the evidence of bees are the primary pollinator of flowering plants at low, middle, and high altitudes in Taiwan.

Hymenopteran Color Space Modeling and Descriptors of Floral Colors

To analyze the FCD as perceived by bees, we used the bee color hexagon model which is widely accepted in relevant studies (Chittka, 1992; Chittka and Menzel, 1992; Dyer et al., 2012; Shrestha et al., 2014, 2019b; Kantsa et al., 2018; Kemp et al., 2019). In this model, the reflectance spectrum of each species is translated into a locus in a two-dimensional plane of the hexagon color space (Chittka, 1992) by employing photoreceptor sensitivities for a bee (350, 440, and 540 nm) (Dyer, 1999), standard D65 illumination (Judd et al., 1964) corrected for photon flux and assuming the visual system was adapted to the leaf-green background (reflectance spectra of the leaves for all samples in our study, see **Supplementary Method S1**). We categorized hexagon color space in to six sub-sectors (*BLUE*: B; *BLUE GREEN*: BG; *GREEN*: G; *UV-GREEN*: UG; *ULTRAVIOLET*: UV; *UV-BLUE*: UB) following the method used in Chittka et al. (1994). Each color locus in bee hexagon was also specified as Polar co-ordinate (a certain vector angle θ and vector magnitude r) or Cartesian co-ordinate (a certain x and y) (Chittka et al., 1994).

To quantitatively compare the overall FCD among different geographic regions and different altitudinal regions, we calculated the area of the minimum convex polygon (MCP) which encapsulate all color loci using the function 'Polygon' in R package SP (version no. 3.4.4).

For descriptors of floral spectral signals considering bee color vision, color hue can be defined as the vector angle θ of color loci in bee hexagon, while color contrast can be defined as the vector magnitude r from the achromatic center of color space that represents background information to the locus of a color (Chittka, 1992). Color distance is the Euclidean distance (Cartesian co-ordinate) between two color loci in bee hexagon (Chittka, 1992; Garcia et al., 2017).

Phylogenetic Tree

To access phylogenetic-informed analysis for our three altitudinal group data, we constructed the phylogenetic tree for all species using Phylomatic 3.0 (Webb and Donoghue, 2005), which was widely used in the multispecies analysis (Stournaras et al., 2013; Shrestha et al., 2014; Zanne et al., 2014; Ohashi et al., 2015). We used Wikström et al. (2001, 2015) to calibrate the many of the nodes and used other published references whereas our genus label phylogeny were based on Soltis et al. (2011) (see details in **Supplementary Table S3**). In the output phylogenetic tree, relationship among all family level and some genera level clades (e.g., in Rosaceae and Fabaceae) were left as unresolved

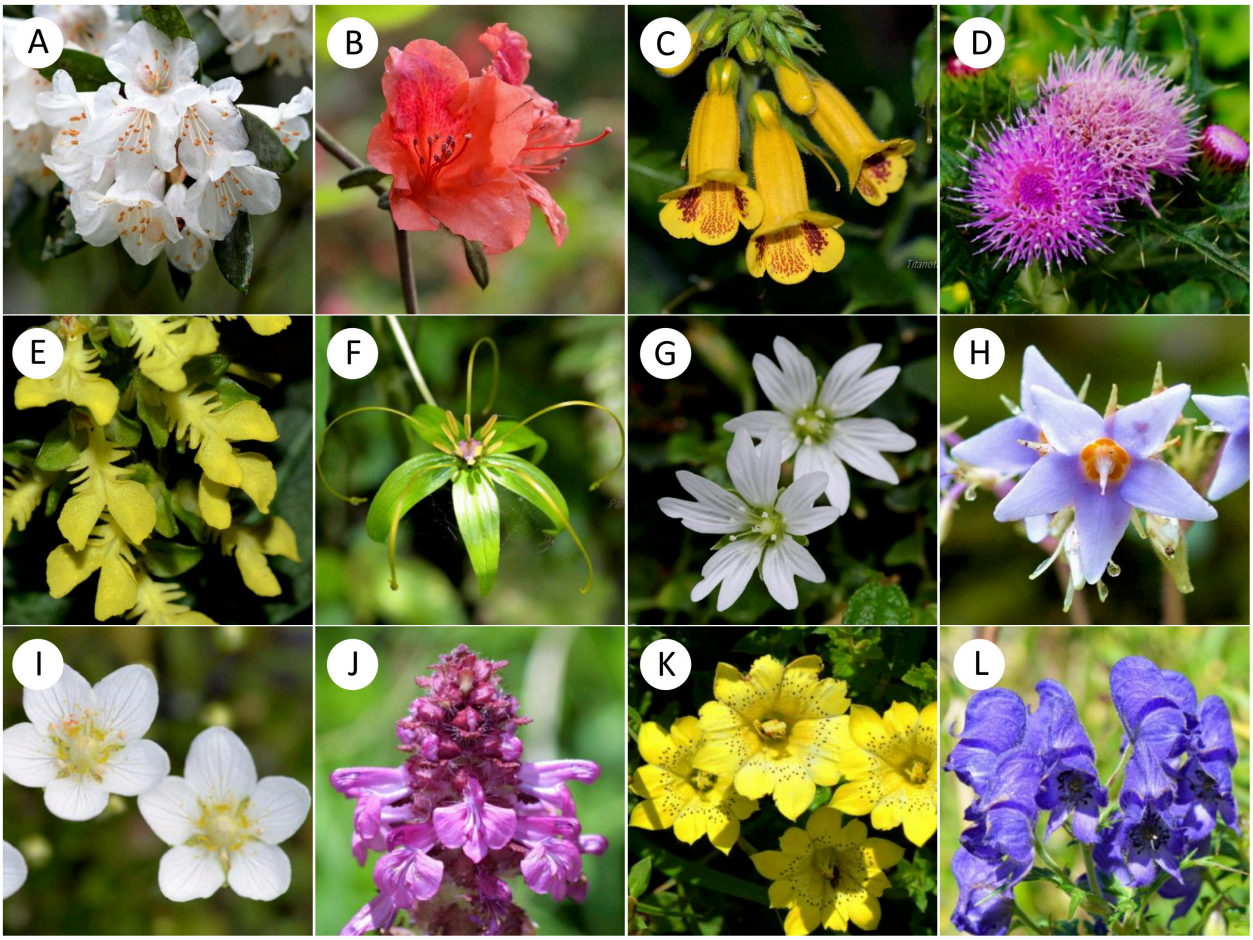


FIGURE 2 | Examples of representative native flowering species from low **(A–D)**, middle **(E–H)** and high **(I–L)** altitudes of Taiwan. **(A)** *Rhododendron formosanum*; **(B)** *Rhododendron oldhamii*; **(C)** *Titanotrichum oldhamii*; **(D)** *Cirsium japonicum* var. *australe*; **(E)** *Odontochilus bisaccatus*; **(F)** *Paris polyphylla* var. *stenophylla*; **(G)** *Stellaria arisanensis*; **(H)** *Conandron ramondioides*; **(I)** *Parnassia palustris*; **(J)** *Pedicularis verticillata*; **(K)** *Gentiana scabrida* var. *punctulata*; **(L)** *Aconitum fukutomei*.

polytomies (**Figure 3**). A phylogenetic distance of each pairwise species was computed with function 'cophenetic.phylo' in R package APE. The output tree Appendix A available in *Dryad Digital Repository* (Tai et al., 2020).

Floral Color Assembly (FCA)

We used mean-pairwise distance (MPD) for the color loci in bee hexagon, which we termed MPD_{color}, to evaluate the pattern of FCA in low, middle and high altitudes. First, we calculated the actual mean pairwise distance (actual MPD_{color}) for all color loci of each altitude. This actual MPD_{color} will be compared with the mean of nulls MPD_{color} (indicating random assembly), which is generated from randomly resampling the color loci from species pool for 1,000 times. Species pool comprises all samples in our study and the sample size for each resampling is identical to the species number within that region.

The pattern of FCA is determined by the comparison between actual MPD_{color} and the mean of nulls MPD_{color}. We computed all MPD_{color} and its significance *p*-value with function 'ses.mpd' in R package PICANTE (Kembel et al., 2010) (details provided in **Supplementary Method S2**).

Phylogenetic Structure

We used mean-pairwise distance (MPD) to evaluate the pattern of phylogenetic structure of species in low, middle and high altitudes, except that phylogenetic distance rather than color distance is the variable analyzed here (thus termed MPD_{phylo}). The pattern of phylogenetic structure was determined by the same procedure of comparison between actual MPD_{phylo} and the mean of nulls MPD_{phylo} as that of FCA described above. That the actual MPD_{phylo} was lower than the mean of nulls MPD infers species in that region tend to be closely related to each other (phylogenetic cluster). On the contrary, if actual MPD_{phylo} was larger than the mean of nulls MPD_{phylo} this would infer that species tend to be distantly related to each other (phylogenetic over-dispersion). Likewise, higher *p*-value indicates stronger phylogenetic over-dispersion, while lower

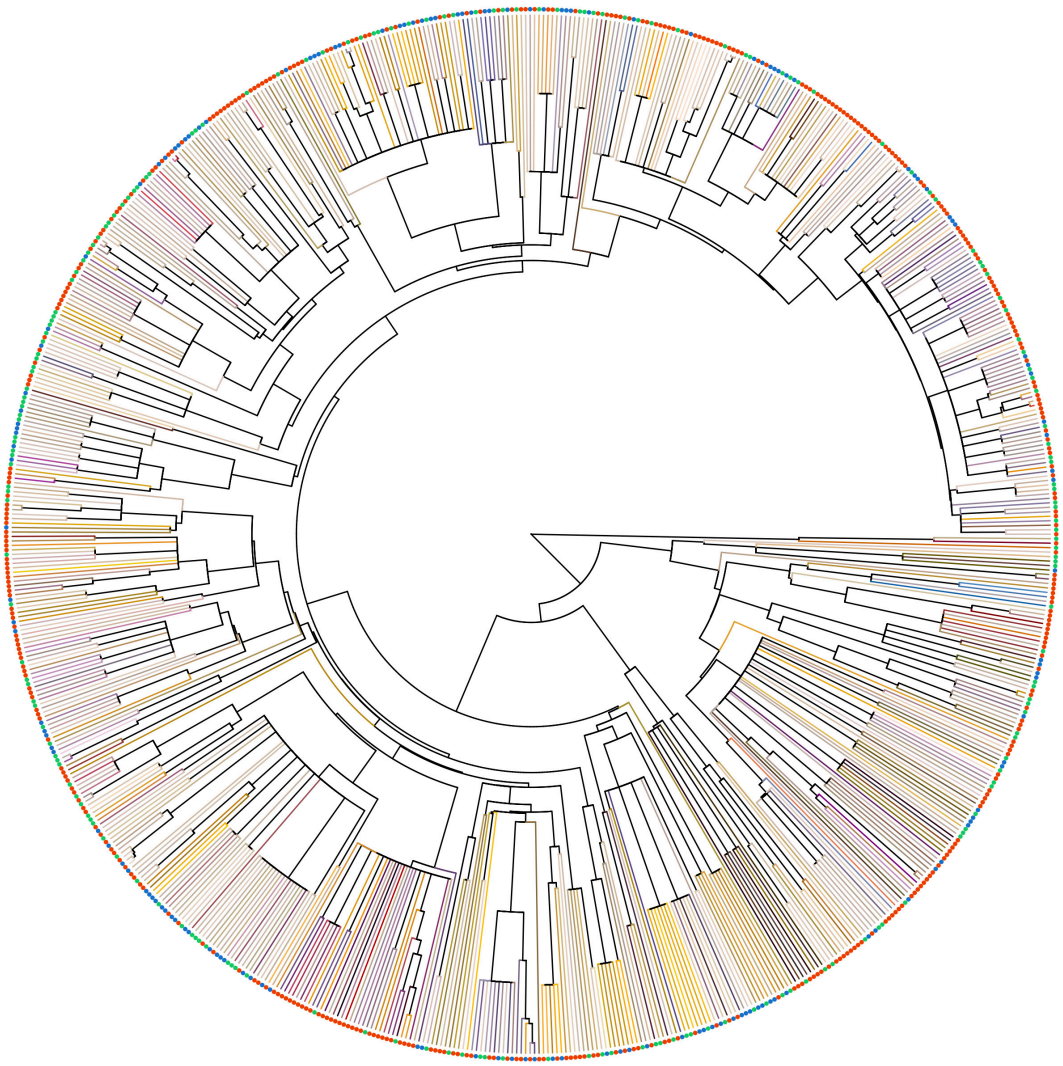


FIGURE 3 | Phylogenetic relationship of the species in our samples. Terminal branches were painted as the floral color of each species in human vision, which was generated from the reflectance spectrum with function 'spec2rgb' of R package PAVO. Solid circles at the tip represents the altitude region of the species (red: low-altitude, green: middle-altitude, blue: high-altitude).

p-value indicates phylogenetic cluster. We used the PICANTE function ses.mpd for these calculations (Kembel et al., 2010). In addition, we also calculated mean nearest taxon distance ($MNTD_{phylo}$) to evaluate the phylogenetic structure and details for these metrics are given in the **Supplementary Method S2**.

Phylogenetic Signal

We examined phylogenetic signal with the commonly used Blomberg's K in previous studies (Blomberg et al., 2003; Muchhala et al., 2014; Shrestha et al., 2014). We calculated the Blomberg's *K* values of the two key floral color descriptors: color hue (Shrestha et al., 2014), and color contrast (referred to as saturation in some studies) in bee hexagon (Lunau, 1990; Kantsa et al., 2017) of low, mid and high-altitude floras in our study using the function 'phylosig' in R package PHYTOOLS (Revell, 2012). We also performed Mantel test to evaluate the phylogenetic signal (Muchhala et al., 2014; Shrestha et al., 2014; Bergamo et al., 2018). Additional details of the calculations for Blomberg's *K* and Mantel test are given in the **Supplementary Method S3**.

RESULTS

Among 727 flowering plant species, the most common floral color in human vision was white (45%), followed by yellow (18%), pink (13%), and purple (11%) whereas species with green (4%), blue (3%), red (3%), and other/orange (3%) flowers were less frequent in Taiwan. Our analysis of their reflectance spectra showed that almost all white flowers are UV-absorbed white (e.g., **Figure 4b**), consistent with previous studies (Inouye and Pyke, 1988; Dyer, 1996; Kevan et al., 1996). Spectra of yellow flowers could be

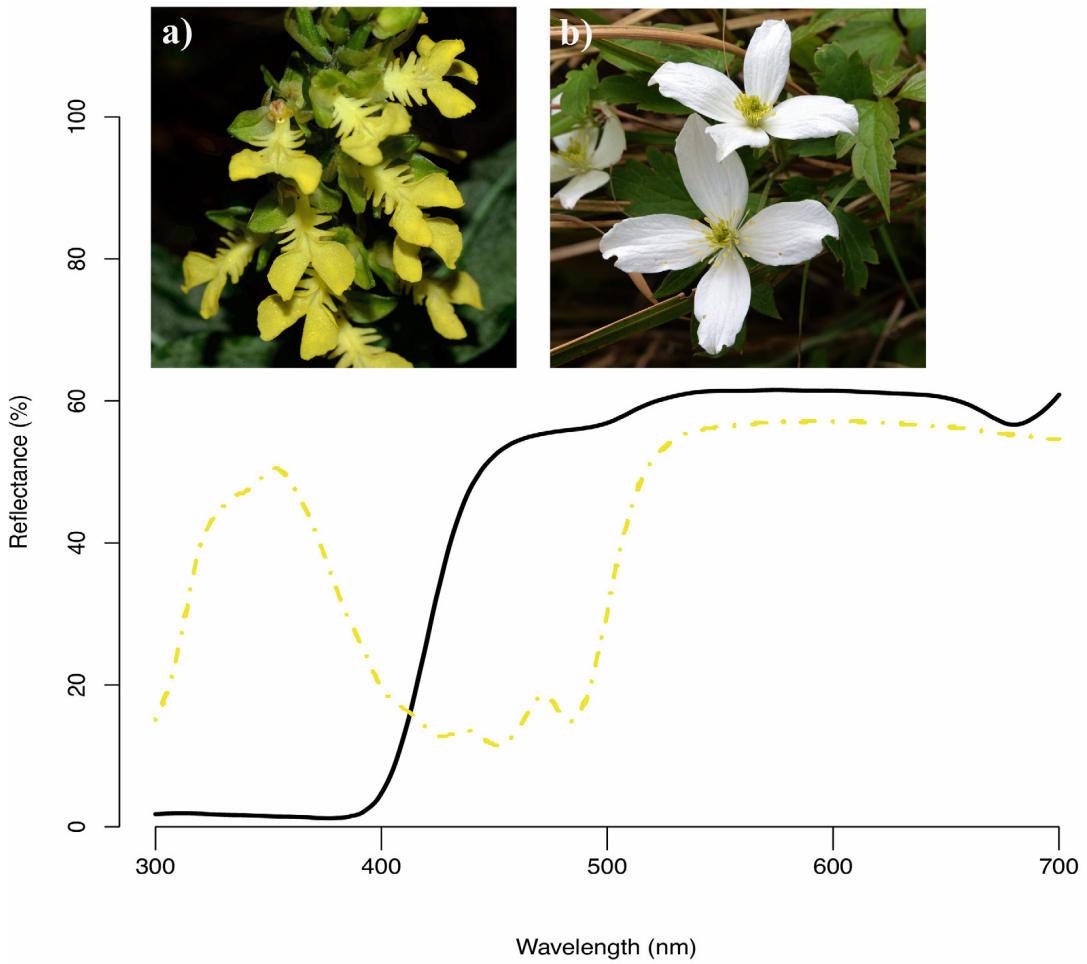


FIGURE 4 | Example reflectance spectra of two native species in our study **(a)** *Odontochilus bisaccatus* (dash yellow curve, human yellow) and **(b)** *Clematis montana* (black line curve, human white).

categorized into two types (Dyer, 1996) based on the presence of UV (67%, e.g., **Figure 4a**) and absence UV (33%) reflection.

Floral Color Diversity (FCD)

We used bee color space to evaluate the pattern of FCD in Taiwan (TW) and compared the patterns to that of the continental island of Australia (AUS), the mountains of Nepal (NPL) and to Japan (JPN) (temperate continental islands) (**Figures 5A–D**). Color loci of floras in TW covered a broad range in bee hexagon (**Figure 5A**), which was comparable to that of continental floras from AUS (**Figure 5C**) and mountains of NPL (**Figure 5D**). The Area of MCP (representing FCD) of color loci in TW was 0.558 (hexagon squared units) which was larger than that (0.498) for AUS, but lower than that (0.783) for both NPL and JPN 0.588 (details **Supplementary Table S5**), thus suggesting overall a somewhat similar pattern to what has been previously observed.

The color loci of floras in TW concentrated to two distinct clusters in bee hexagon. One cluster has the greatest frequency toward the bee-BG vertex of bee hexagon, which can be termed 'White Arm' because most floral colors inside were human white (**Figure 5A**). Similarly, the other cluster lay toward bee-UG vertex can be termed 'Yellow Arm' as it was made up of human yellow flowers (**Figure 5A**). We also found similar 'White and Yellow Arms' in the pattern of JPN (**Figure 5B**), and these colors are also frequent flower hues from AUS (**Figure 5C**) and the mountains of NPL (**Figure 5D**), although the "arm" is less distinct.

We evaluated the variation of FCD between low, middle, and high altitudes in Taiwan by comparing the area of MCP of the color loci in bee hexagon (**Figure 6**). We found the area of MCP of low-altitude (0.546) was larger than that in high-altitude (0.304) and almost twice as that of middle-altitude (0.270). It was possible that the unequal sample size between different altitudes (from low to high: n = 399, 186 vs. 142) may bias the areas of MCP. We thus conducted permutation test to resolve this argument, and the result revealed that the areas of MCP of middle and high altitudes persisted to be lower than that of low-altitude (**Supplementary Method S4** and

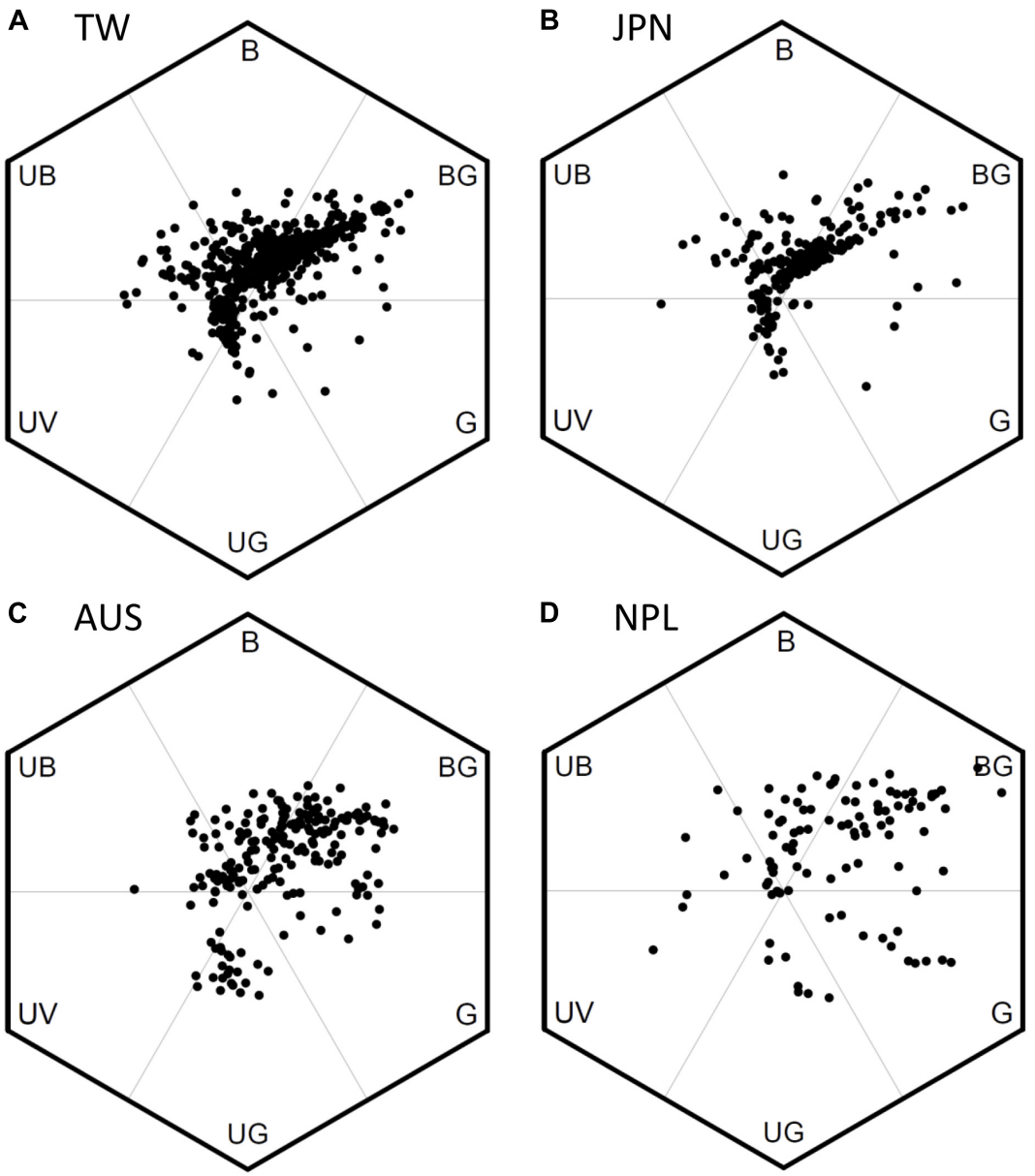


FIGURE 5 | Bee color hexagon showing the floral color loci of species from four separate geographic regions. **(A)** 727 species of Taiwan; **(B)** 212 species of Japan (Makino and Yokoyama, 2015); **(C)** 201 species of Australia (Dyer et al., 2012; Burd et al., 2014); **(D)** 107 species of Nepal (Shrestha et al., 2014). We recalculated the color loci in bee hexagon of Japanese samples based on the published reflectance spectra in Makino and Yokoyama (2015).

Supplementary Figure S2). Thus, this result suggested that floras of low-altitude in Taiwan exhibited greater FCD than that of middle and high altitudes.

Color Hexagon Sector

The most predominant peak of absolute frequency for floral color loci of all Taiwanese floras (i.e., from low to high altitudes) in bee hexagon sector occurred around 60°, which lies on the bee's dominant preference regions (**Figure 7**). In addition, when compared to the global floral color distribution, Taiwanese data exhibited no other distinct peaks outside the bee preference regions.

Floral Color Assembly (FCA)

In order to test if floral colors in Taiwan show evidence of either over-dispersion or clustering, we compared the actual mean pairwise distance (MPD_{color}) of the color loci in bee hexagon to the nulls MPD_{color} based on random assembly. We found the actual MPD_{color} of high-altitude species (0.245) was larger than that (0.227) of the mean of nulls MPD_{color} (random assembly), and its p-value was 0.930 (**Table 1**). This suggested that the floral

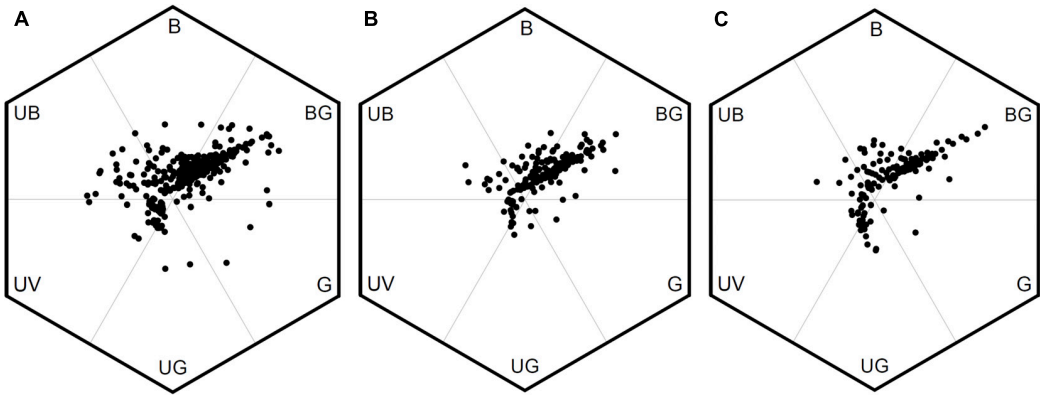


FIGURE 6 | Bee color hexagon showing floral color loci of species from low, middle, and high altitudes in Taiwan. **(A)** 399 species from low-altitude, **(B)** 186 species from middle-altitude, and **(C)** 142 species from high-altitude.

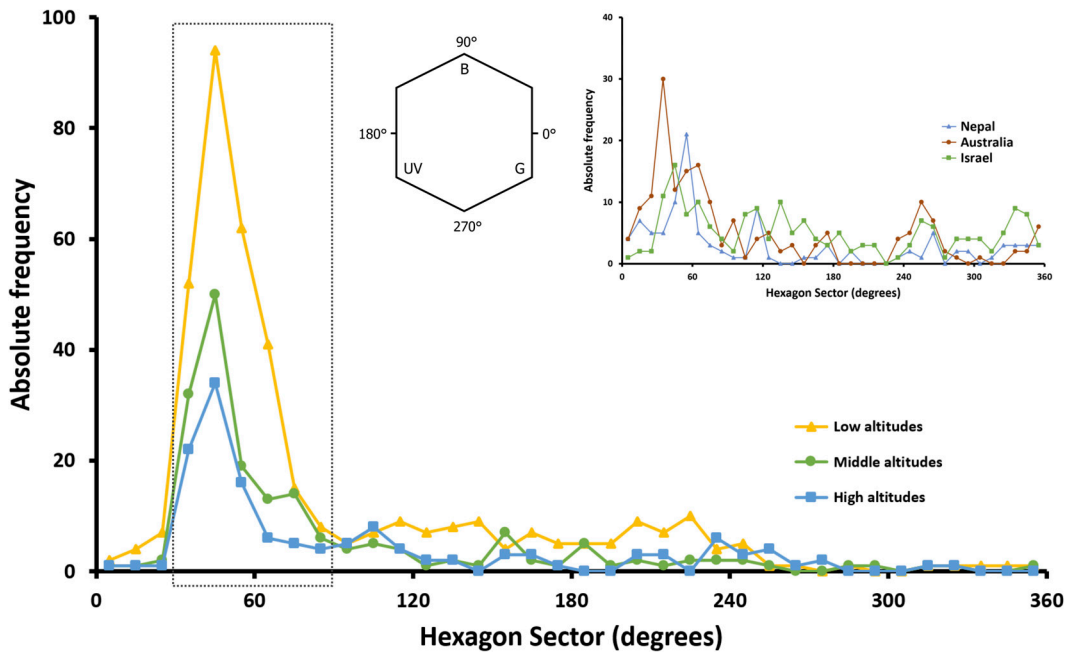


FIGURE 7 | Absolute frequency distribution of floral color loci in 10° sector of bee color hexagon as proposed by Chittka et al. (1994). Distributions for species from low, middle and high altitudes in Taiwan (yellow, green and blue symbols and lines) are plotted, and compared to the global floral color distribution in hexagon color space (inset). The inset figure shows the distribution of species across the Australian continent (brown symbols and line; data from Dyer et al., 2012; Shrestha et al., 2019b), along sub-tropical and sub-alpine transects of the Nepalese Himalayas (blue symbols and line; data from Shrestha et al., 2014) and Israel (green symbols and line; data from Chittka et al., 1994). The dotted rectangle encloses the bee-blue and bee-blue–green sectors of the color space (30°–90°, see inset) where honeybees (Giurfa et al., 1995), bumblebees (Raine and Chittka, 2005) and the stingless bee *Tetragonula carbonaria* (Dyer et al., 2016) all demonstrate their strongest innate color preference.

colors of high-altitude species may have diverged from each other, i.e., floral color over-dispersion. On the other hand, floral colors of middle-altitude species are significantly clustered ($p = 0.040$), whereas MPD_{color} of low-altitude species appeared to be best explained by a random assembly ($p = 0.599$) (**Table 1**).

Given that not all plant species in high-altitude were pollinated by bees as other insect pollinators like flies are relatively more frequent in cooler environments (Totland, 1993; Arnold et al., 2009b; Lord et al., 2013; Paudel et al., 2016; Shrestha et al., 2016), we specifically evaluated the FCA of the species visited by either bees or other insects (see **Supplementary Method S5**). If the calculated MPD_{color} and relative p-value increase, this indicates that high-altitude plant species associating with the bees or other insects exhibit more divergent floral colors. Indeed, we found that the MPD_{color} and relative p-values of both bee visit ($MPD_{color} = 0.260$, $p = 0.950$) and insect visit ($MPD_{color} = 0.267$, $p = 0.980$) species in high-altitude has increased (**Table 1**), showing that floral colors exhibited

TABLE 1 | Summary of floral color assembly (FCA) for three altitudes zones in Taiwan considering bee visit species and other insect visit species in high-altitude.

			Mean pairwise distance (MPD_{color})		
		N	**Actual**	**Nulls (mean ± sd)**	**P**
Among altitudes	Low	399	0.228	0.227 ± 0.005	0.599
	Middle	186	0.209	0.227 ± 0.010	0.040
	High	142	0.245	0.227 ± 0.012	0.930
High-altitude species	Bee-visit	41	0.260	0.227 ± 0.024	0.950
	Insect-visit	66	0.267	0.227 ± 0.019	0.980

P-value is determined by the rank of the actual MPD_{color} in 1000 nulls MPD_{color}. Euclidean distance of each pairwise color loci in bee hexagon were the distance analyzed here.

stronger over-dispersed within high-altitude species visited by bee and insect group.

Phylogenetic Structure

To clarify if relatedness of species accounted for the floral color over-dispersion within high-altitude plant community, we analyzed the comparative phylogenetic structure of species in the low, mid and high-altitude zones by calculating the mean pairwise distance (MPD_{phylo}) and mean nearest taxon distance ($MNTD_{phylo}$) (details in **Table 2**). Both actual MPD_{phylo} and $MNTD_{phylo}$ for species in low-altitude were close to the mean of nulls MPD_{phylo} and $MNTD_{phylo}$ (indicative of random assembly), and the *p*-values (0.639 and 0.645) also suggested that the phylogenetic structure of low-altitude species approached to random assembly (**Table 2**). The MPD_{phylo} for middle-altitude species was close to the mean of nulls MPD_{phylo} (p = 0.540), whereas the $MNTD_{phylo}$ was much lower than the mean of nulls $MNTD_{phylo}$ (p = 0.098) (**Table 2**). These revealed that middle-altitude species were randomly assembled across the entire phylogenetic tree, but clustered toward the tips of the phylogenetic tree. Further, we found the MPD_{phylo} value of high-altitude species (260.6) was lower than the mean of nulls MPD_{phylo} (267.0) (p = 0.174), this indicated that high-altitude species are more closely related to each other across the entire phylogenetic tree (**Table 2**). Additionally, actual $MNTD_{phylo}$ of high-altitude species was significantly lower than that of the mean of nulls $MNTD_{phylo}$ (p = 0.004) (**Table 2**), which supported the evidence that high-altitude species were significantly clustered toward the tips of the phylogenetic tree.

TABLE 2 | Summary of the phylogenetic structure of low, middle and high-altitude species in Taiwan.

	Altitude	***N***	**Actual**	**Nulls (mean ± sd)**	**P**
MPD_{phylo}					
	Low	399	268.1	267.0 ± 3.0	0.639
	Middle	186	267.8	267.1 ± 5.7	0.54
	High	142	260.6	267.0 ± 6.8	0.174
$MNTD_{phylo}$					
	Low	399	94	93.3 ± 2.1	0.645
	Middle	186	106.4	111.8 ± 4.2	0.098
	High	142	105.3	118.9 ± 5.0	0.004

The P-value is determined by the rank of the actual MPD_{phylo} (or $MNTD_{phylo}$) in 1000 nulls MPD_{phylo} (or $MNTD_{phylo}$). Larger P-value indicates stronger phylogenetic over-dispersion, while lower P-value indicates stronger phylogenetic cluster. Phylogenetic distance calculated from the phylogenetic tree was the distance analyzed here.

Phylogenetic Signal

The *K* values for two color descriptors (color hue, color contrast) that are known to be key factors in flower signaling were significantly deviated from random expectation (K = 0) and lower than that expected by Brownian motion evolution (K = 1) at low and high-altitude (**Table 3**). As all *K* values of low and high-altitudes (0.275∼0.454) were closer to zero than one, this suggested that the key descriptor values were more divergent to each other among closely related species in low and high-altitudes (i.e., weak phylogenetic signal). Moreover, Mantel test of correlation supported this weak phylogenetic signal at high altitude (r = 0.008, p = 0.417). This suggested that floral colors are liable, thus these closely related species do not necessarily have similar floral colors and may adapt to optimal pollinator in a particular environment. Interestingly, *K* value for color hue (K = 0.629) of middle altitude species did not deviate from 1 (**Table 3**), indicating floral colors of closely related species in middle altitude are more similar as that expected by Brownian motion evolution. Consistently, the Mantel test detected a relatively stronger correlation between phylogenetic distance and color distance of the middle altitude species (r = 0.125, p = 0.005). These data thus revealed a stronger phylogenetic signal, i.e., closely related species in middle-altitude plant community shared more similar floral colors than that in a low and high-altitude plant community.

TABLE 3 | Phylogenetic signal for the floral color descriptors of species in low, mid, and high-altitude of Taiwan.

	Blomberg's *K*			**Mantel correlation**	
Altitude	***K***	***K* = 0**	***K* = 1**	***r***	***p***
Low				0.078	0.013
Color hue	0.283	0.025	0.003		
Color saturation	0.275	0.017	0.001		
Middle				0.125	0.005
Color hue	0.629	0.002	0.186		
Color saturation	0.345	0.041	0.008		
High				0.008	0.417
Color hue	0.454	0.012	0.007		
Color saturation	0.443	0.021	0.018		

For each color descriptor, we calculated Blomberg's K value and tested whether the K value significantly deviated from zero (K = 0, no phylogenetic signal) or from one (K = 1, indicative of Brownian motion evolution) (significance level P < 0.05). Color hue and color saturation are floral color descriptors derived from color loci in bee hexagon.

DISCUSSION

How flowers evolved color signals in different environments is important for understanding the plant–pollinator interactions that have occurred in the past and driven evolutionary processes (Fenster et al., 2004), and what might happen in the future with changing climatic conditions and/or pollinator distributions (Hegland et al., 2009; Shrestha et al., 2018). Based on large scales studies, species in tropical regions have been proposed to exhibit more diverse colors compared to the higher latitudes, and this may result from greater strength of biotic interaction in tropics to accelerate the evolution of coloration (Schemske et al., 2009; Dalrymple et al., 2015). However, previous island studies away from the tropics tend to show low FCD.

In the current study, we considered the uniquely placed island of Taiwan and used a hexagon color space model to plot flower loci and subsequently calculate FCD as the minimum convex polygon (MCP) encapsulating all loci. Our data revealed that the overall FCD of TW was 0.558, which is greater than AUS (0.498), but less than JPN (0.588) or NPL (0.783). Thus, despite the species richness of a mountainous tropical island with close proximity to mainland plant sources that we hypothesized should lead to high color diversity within Taiwan, it appears the data falls in the middle of FCD values for our comparative country data sets. We found that ca. 33% of the floras in our collected TW samples are endemic species, which have MCP (0.400) (**Supplementary Table S4**) and accounted for 72% of the overall FCD in TW, suggesting that flower color signaling is very consistent to our comparative data sets from different parts of the world. We also observed that within TW there was evidence of changes in color signaling with increasing altitude as considering low (0–900 m a.s.l.), middle (1500–2200 m a.s.l.) and high-altitude regions (2800–3300 m a.s.l.) the respective FCD values were 0.546 (399 species), 0.270 (186 species) and 0.304 (142 species) (**Figure 6**). Such a change did not appear to be caused simply by the number of species since at high-altitude there was a higher FCD, but actually a lower number of plant species compared to the mid-altitude region.

Taiwan is an atypical island as its low isolation level allows plant species to readily migrate to TW from subtropical Asia (e.g., China) and tropical Asia (e.g., Philippines) (Hsieh, 2002; Huang, 2011), and there is ample habitat diversity to host a range of potential pollinators. The co-shared floras covered 95% of the total FCD in TW (**Supplementary Table S4**). Our results suggest that observed differences in FCD at different altitudes may be mediated by lineage diversity, rather than plant species richness. The evidence for this is threefold. Firstly, we found FCD in low altitudes was greater than that in middle and high altitudes. This was because some particular color floras belonging to the families, e.g., Aristolochiaceae, Zingiberaceae, Fabaceae and Rubiaceae (*Mussaenda*) (**Figure 3**), were common in low altitudes but less frequent or absent in middle and high altitudes, and the color loci the flowers of these plants were often distributed toward the outer regions of hexagon color space resulting in an increase the FCD at low altitudes (**Figure 6**). Secondly, the phylogenetic structure of low-altitude floras was of over-dispersed, whilst the phylogenetic structure of high-altitude floras showed evidence of being clustered (**Table 2**). This implies that lineages of low-altitude floras contain more diverse groups than that of high-altitude floras do. Thirdly, the results of our permutation test revealed that the greater FCD in low altitude was neither due to the larger sample size, nor species richness (**Supplementary Figure S2**). When considering lineage diversity as the key explanation for why FCD changed with altitude, our finding is consistent with the findings of previous studies in Norway and Nepal as the lineage diversity could be represented by the amount of family and genera. The greater FCD in low altitudes of Norway was linked to greater lineage diversity (24 families, 47 genera) than in high altitudes (13 families, 14 genera) (Bergamo et al., 2018) (**Supplementary Table S5**). Similarly, as lineages are more diverse in flowering samples from high altitude (25 families, 45 genera) than that from low altitude (17 families, 38 genera) of Nepal, greater FCD was instead observed in high altitude (Shrestha et al., 2014) (**Supplementary Table S5**). Taken together these comparative findings suggest that lineage diversity is a key factor mediating FCD.

Lázaro et al. (2008) reported that flowers of white and yellow appearance to a human observer are represented by a more diverse range of plant species when considering the total number of plant species in an environment. Color trapping experiments in alpine Australia (Pickering and Stock, 2003) found that white and yellow flowers were the top two choices for multiple pollinators. Interestingly we also found that human white (bee BLUE-GREEN) and yellow (bee UV-GREEN) flowers were more dominant in Taiwan (**Figure 5A**). White and yellow flowers are also dominant on other islands like the temperate island of Japan (Makino and Yokoyama, 2015), some islands and mountains of southern Hemisphere (Webb and Kelly, 1993; Bernardello et al., 2001; Bischoff et al., 2013b), and these colors are also the most frequent flower hues on larger continental studies (Dyer, 1996; Arnold et al., 2009a; Dyer et al., 2012; Shrestha et al., 2014). An exception case is the study of MI (Shrestha et al., 2016) where no bees have ever been present, and the flowers of MI show a distinctive long wavelength yellowish color that fits with known long wavelength color preferences of flies; the only animal pollination vector on MI. Similarly, orchids on mainland Australia that are pollinated by flies show a distinctive long-wavelength reflection, whilst bee-pollinated orchids from the same community environment display color signals close to the spectral profiles of bee pollinated flowers around the world (Shrestha et al., 2019a). Thus, color preferences of insect pollinators may be a driving factor of flower color signaling. Such spectra fit with measured color preferences in major bee genera like *Apis* sp. and *Bombus* sp., as well as phylogenetically separated bee species like the stingless bee *Tetragonula carbonaria* (**Figure 7**). Interestingly, such color preferences have been observed to be biologically relevant considering the foraging decisions of naïve bees visiting real flowers (Dyer et al., 2019), providing a plausible explanation for how insect preferences do influence plant fitness and flower color signaling (Giurfa et al., 1995; Shrestha et al., 2020). In addition, floral color loci in the three altitudinal regions of TW we tested consistently and predominantly fall in the sector of bee color space preferred by bees (**Figure 7**). The increased FCD at higher compared to

middle altitudes in TW suggests that the increased frequencies of alternative pollinators like flies may promote some diversification of flower color signaling, and future work could seek to test plant–pollinator networks to understand how signaling may be influenced by different insects with communities (Kantsa et al., 2017; Shrestha et al., 2019a,b).

Floral color over-dispersion has been reported for some alpine plant communities around the world (McEwen and Vamosi, 2010; Shrestha et al., 2014). Our result was in agreement with McEwen and Vamosi (2010) who found four of five alpine plant communities in Canada exhibited a trend towards floral color over-dispersion. Similarly, Shrestha et al. (2014) reported floral colors were significantly over-dispersed in high altitudes, whilst tending to be clustered at low altitudes in Nepal. Our results are also consistent with previous studies which reported the bees have driven the floral color over-dispersion within alpine plant communities in Nepal (Shrestha et al., 2014) and contribute to the floral color diversification of sympatric *Pedicularis* sp. in Hengduan Mt. (China) (Eaton et al., 2012).

Based on our phylogenetic-informed analysis, we found high altitude floras more closely related to each other than expected by the random assembly (i.e., phylogenetic cluster) (**Table 2**). However, floral color of these related species actually exhibited higher divergence than that predicted by neutral Brownian motion evolution (i.e., weak phylogenetic signal) (**Table 3**). Consistently, a community-based study in Nepal (Shrestha et al., 2014), and a clade-based study in Andean Solanaceae (Muchhala et al., 2014) also reported that floral color over-dispersion was mainly attributed to the divergent floral colors among sympatric closely related species.

Previous studies suggest that floral color over-dispersion most typically occurs through either character displacement or competitive exclusion (Emerson and Gillespie, 2008; Sargent and Ackerly, 2008), although it is often difficult to distinguish these two ecological processes at community level. In the current study if floral color over-dispersion had resulted from competitive exclusion, we will expect that closely related species are not likely to co-exist as they would compete with each other, thus the phylogenetic structure of the community should tend to be over-dispersed (Cavender-Bares et al., 2004; Sargent and Ackerly, 2008). On the other hand, if character displacement occurred, closely related species should evolve divergent floral colors perhaps due to reduce competition for pollinators, therefore a weak phylogenetic signal is expected (Revell et al., 2008; Muchhala et al., 2014). We found that the high altitudes floras tend to be phylogenetically clustered rather than over-dispersed (**Table 2**), and their floral colors exhibited weak phylogenetic signal (**Table 3**) which is consistent with character displacement that facilitated the co-existence of similar species. In addition, our comparative results suggest that bees appear to be the major pollinator in Taiwan consistent with other large studies around the world (**Supplementary Figure S1**) where the most frequently observed flower colors fit with the innate preferences of bees (**Figure 7**). Thus results from a large tropical–subtropical island reveal flower color evolution around the world appears to have most frequently followed a similar trajectory.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. Dryad Dig. Repos. https://doi.org/10.5061/dryad.63xsj3v08.

AUTHOR CONTRIBUTIONS

E-CY, C-NW, MS, and AD developed the concept. K-CT, E-CY, and C-NW collected the data. MS and AD contributed data from Australia and Nepal. K-CT, MS, AD, and C-NW did data analysis and drafted the final version of the manuscript. All authors contributed to review the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.582784/full#supplementary-material

Supplementary Figure 1 | Marker point calculation.

Supplementary Figure 2 | Frequency distribution of the areas of MCP in permutation test.

Supplementary Table 1 | Detailed information of the sampling sites.

Supplementary Table 2 | Details of the list of the 727 flowering plant species in our current study, including the altitude and location is provided is ".csv" file.

Supplementary Table 3 | Studies used to provide subfamilial topology in the phylogenetic tree.

Supplementary Table 4 | Summary of the percentage for species in our sample co-sharing with other geographic region.

Supplementary Table 5 | FCD for Nepal, Norway, Japan, and Australia.

Supplementary Method 1 | Details of floral color measurement.

Supplementary Method 2 | Details of MPD_{color} and $MNTD_{phylo}$ for phylogenetic structure.

Supplementary Method 3 | Details of the Blomberg's *K* and Mantel test.

Supplementary Method 4 | Procedure of permutation test.

Supplementary Method 5 | Identification of flower visitors of high-altitude species.

REFERENCES

Adams, J. M., Kang, C., and June-Wells, M. (2014). Are tropical butterflies more colorful? *Ecol. Res.* 29, 685–691. doi: 10.1007/s11284-014-1154-1

Arnold, S. E. J., Le Comber, S. C., and Chittka, L. (2009a). Flower color phenology in European grassland and woodland habitats, through the eyes of pollinators. *Israel J. Plant Sci.* 57, 211–230. doi: 10.1560/ijps.57.3.211

Arnold, S. E. J., Savolainen, V., and Chittka, L. (2009b). Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. *Arthropod Plant Interact.* 3, 27–43. doi: 10.1007/s11829-009-9056-9

Bergamo, P. J., Telles, F. J., Arnold, S. E. J., and de Brito, V. L. G. (2018). Flower colour within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-5

Bernardello, G., Anderson, G. J., Stuessy, T. F., and Crawford, D. J. (2001). A survey of floral traits, breeding systems, floral visitors, and pollination systems of the angiosperms of the Juan Fernández Islands (Chile). *Bot. Rev.* 67, 255–308. doi: 10.1007/BF02858097

Bingham, R. A., and Orthner, A. R. (1998). Efficient pollination of alpine plants. *Nature* 391, 238–239. doi: 10.1038/34564

Biq, C.-C., Shyu, C.-T., Chen, J.-C., and Boggs, S. (1985). "Taiwan: geology, geophysics, and marine sediments," in *The Ocean Basins and Margins*, eds A. E. M. Nairn, F. G. Stehli, and S. Uyeda (Boston, MA: Springer), 503–550. doi: 10.1007/978-1-4613-2351-8_11

Bischoff, M., Campbell, D. R., Lord, J. M., and Robertson, A. W. (2013a). The relative importance of solitary bees and syrphid flies as pollinators of two outcrossing plant species in the New Zealand alpine. *Austr. Ecol.* 38, 169–176. doi: 10.1111/j.1442-9993.2012.02389.x

Bischoff, M., Lord, J. M., Robertson, A. W., and Dyer, A. G. (2013b). Hymenopteran pollinators as agents of selection on flower colour in the New Zealand mountains: salient chromatic signals enhance flower discrimination. *N. Z. J. Bot.* 51, 181–193. doi: 10.1080/0028825X.2013.806933

Blomberg, S. P., Garland, T., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1554/0014-38202003057[0717:TFPSIC]2.0.CO2

Bradshaw, H. D., and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Brooks, T. M., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B., Rylands, A. B., Konstant, W. R., et al. (2002). Habitat loss and extinction in the hotspots of biodiversity. *Conserv. Biol.* 16, 909–923. doi: 10.1046/j.1523-1739.2002.00530.x

Buchmann, S. L. (1994). "Diversity and importance of native bees from the Arizona Mexico Madrean Archipelago," in *Biodiversity and Management of the Madrean Archipelago : The Sky Islands of Southwestern United States and Northwestern Mexico*, eds L. F. de Bano, G. J. Gottfried, R. H. Hamre, C. B. Edminster, P. F. Ffolliott, and A. Ortega (Tucson, ARI: DIANE Publishing), 301–310.

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B Biol. Sci.* 281:20132862. doi: 10.1098/rspb.2013.2862

Camargo, M. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L., and Morellato, L. P. (2019). How flower colour signals allure bees and hummingbirds: a community level test of the bee avoidance hypothesis. *N. Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Cavender-Bares, J., Ackerly, D. D., Baum, D. A., and Bazzaz, F. A. (2004). Phylogenetic overdispersion in floridian oak communities. *Am. Natural.* 163, 823–843. doi: 10.1086/386375

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/bf00188925

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of hymenoptera. *Vis. Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Chittka, L., Thomson, J. D., and Waser, N. M. (1999). Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86, 361–377. doi: 10.1007/s001140050636

Cox, P. A., and Elmqvist, T. (2000). Pollinator extinction in the pacific islands. *Conserv. Biol.* 14, 1237–1239. doi: 10.1046/j.1523-1739.2000.00017.x

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2015). Birds, butterflies and flowers in the tropics are not more colourful than those at higher latitudes. *Glob. Ecol. Biogeogr.* 24, 1424–1432. doi: 10.1111/geb.12368

de Jager, M. L., Dreyer, L. L., and Ellis, A. G. (2011). Do pollinators influence the assembly of flower colours within plant communities? *Oecologia* 166, 543–553. doi: 10.1007/s00442-010-1879-7

de Salas, M. F., and Baker, M. L. (2015). *A Census of the Vascular Plants of Tasmania, Including Macquarie Island*. Hobart, TAS: Tasmanian Herbarium, Tasmanian Museum and Art Gallery.

Dyer, A. G. (1996). Reflection of near-ultraviolet radiation from flowers of australian native plants. *Austr. J. Bot.* 44, 473–488. doi: 10.1071/bt9960473

Dyer, A. G. (1999). Broad spectral sensitivities in the honeybee's photoreceptors limit colour constancy. *J. Comp. Physiol. A* 185, 445–453. doi: 10.1007/s003590050405

Dyer, A. G. (2006). Discrimination of flower colours in natural settings by the bumblebee species *Bombus terrestris* (Hymenoptera: Apidae). *Entomol. Gen.* 28, 257–268. doi: 10.1127/entom.gen/28/2006/257

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Garcia, J. E., van der Kooi, C., and Wong, B. B. M. (2019). Colour preferences of *Tetragonula carbonaria* Sm. stingless bees for colour morphs of the Australian native orchid *Caladenia carnea*. *J. Comp. Physiol. A* 205, 347–361. doi: 10.1007/s00359-019-01346-0

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016). Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, A. G., Paulk, A. C., and Reser, D. H. (2011). Colour processing in complex environments: insights from the visual system of bees. *Proc. R. Soc. B Biol. Sci.* 278, 952–959. doi: 10.1098/rspb.2010.2412

Eaton, D. A. R., Fenster, C. B., Hereford, J., Huang, S.-Q., and Ree, R. H. (2012). Floral diversity and community structure in *Pedicularis* (Orobanchaceae). *Ecology* 93, S182–S194. doi: 10.1890/11-0501.1

Emerson, B. C., and Gillespie, R. G. (2008). Phylogenetic analysis of community assembly and structure over space and time. *Trends Ecol. Evol.* 23, 619–630. doi: 10.1016/j.tree.2008.07.005

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path to colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Giurfa, M., Núñez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comp. Physiol. A* 177, 247–259. doi: 10.1007/BF00192415

Gray, M., Stansberry, M. J., Lynn, J. S., Williams, C. F., White, T. E., and Whitney, K. D. (2018). Consistent shifts in pollinator-relevant floral coloration along Rocky Mountain elevation gradients. *J. Ecol.* 106, 1910–1924. doi: 10.1111/1365-2745.12948

Gumbert, A., Kunze, J., and Chittka, L. (1999). Floral colour diversity in plant communities, bee colour space and a null model. *Proc. R. Soc. B Biol. Sci.* 266, 1711–1716. doi: 10.1098/rspb.1999.0836

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A.-L., and Totland, Ø (2009). How does climate warming affect plant-pollinator interactions? *Ecol. Lett.* 12, 184–195. doi: 10.1111/j.1461-0248.2008.01269.x

Hsieh, C.-F. (2002). Composition, endemism and phytogeographical affinities of the Taiwan flora. *TAIWANIA* 47, 298–310. doi: 10.6165/tai.2002.47(4).298

Huang, S.-F. (2011). Historical biogeography of the flora of Taiwan. *J. Natl. Taiwan Museum* 64, 33–63.

Inouye, D. W., and Pyke, G. H. (1988). Pollination biology in the Snowy Mountains of Australia: comparisons with montane Colorado, USA. *Austr. J. Ecol.* 3, 191–210. doi: 10.1111/j.1442-9993.1988.tb00968.x

Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T., et al. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Optic. Soc. Am.* 54, 1031–1040. doi: 10.1364/JOSA.54.001031

Kantsa, A., Raguso, R. A., Dyer, A. G., Olesen, J. M., Tscheulin, T., and Petanidou, T. (2018). Disentangling the role of floral sensory stimuli in pollination networks. *Nat. Commun.* 9, 1041–1041. doi: 10.1038/s41467-018-03448-w

Kantsa, A., Raguso, R. A., Dyer, A. G., Sgardelis, S. P., Olesen, J. M., and Petanidou, T. (2017). Community-wide integration of floral colour and scent in a Mediterranean scrubland. *Nat. Ecol. Evol.* 1, 1502–1510. doi: 10.1038/s41559-017-0298-0

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kemp, J. E., Bergh, N. G., Soares, M., and Ellis, A. G. (2019). Dominant pollinators drive non-random community assembly and shared flower colour patterns in daisy communities. *Ann. Bot.* 123, 277–288. doi: 10.1093/aob/mcy126

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1, 280–284. doi: 10.1016/1360-1385(96)20008-1

Lázaro, A., Hegland, S. J., and Totland, Ø (2008). The relationships between floral traits and specificity of pollination systems in three Scandinavian plant communities. *Oecologia* 157, 249–257. doi: 10.1007/s00442-008-1066-2

Li, C.-F., Chytrý, M., Zelený, D., Chen, M.-Y., Chen, T.-Y., Chiou, C.-R., et al. (2013). Classification of Taiwan forest vegetation. *Appl. Veg. Sci.* 16, 698–719. doi: 10.1111/avsc.12025

Lord, J. M., Huggins, L., Little, L. M., and Tomlinson, V. R. (2013). Floral biology and flower visitors on subantarctic Campbell Island. *N. Z. J. Bot.* 51, 168–180. doi: 10.1080/0028825X.2013.801867

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: flower dummy experiments with bumblebees. *J. Comp. Physiol. A* 166, 827–834. doi: 10.1007/BF00187329

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Makino, T. T., and Yokoyama, J. (2015). Nonrandom composition of flower colors in a plant community: mtually different co-flowering natives and disturbance by aliens. *PLoS One* 10:e0143443. doi: 10.1371/journal.pone.0143443

McEwen, J. R., and Vamosi, J. C. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proc. R. Soc. B Biol. Sci.* 277, 2957–2965. doi: 10.1098/rspb.2010.0501

Morawetz, L., Svoboda, A., Spaethe, J., and Dyer, A. G. (2013). Blue colour preference in honeybees distracts visual attention for learning closed shapes. *Journal of Comparative Physiology A* 199, 817–827. doi: 10.1007/s00359-013-0843-5

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in andean Solanaceae. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Oberrath, R., and Böhning Gaese, K. (1999). Floral color change and the attraction of insect pollinators in lungwort (*Pulmonaria collina*). *Oecologia* 121, 383–391. doi: 10.1007/s004420050943

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral colour change in the eyes of pollinators: testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144–1155. doi: 10.1111/1365-2435.12420

Ômura, H., and Honda, K. (2005). Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* 142, 588–596. doi: 10.1007/s00442-004-1761-6

Paudel, B. R., Burd, M., Shrestha, M., Dyer, A. G., and Li, Q.-J. (2018). Reproductive isolation in alpine gingers: how do co-existing *Roscoea* (*R. purpurea* and *R. tumjensis*) conserve species integrity? *Evolution* 72, 1840–1850. doi: 10.1111/evo.13546

Paudel, B. R., Shrestha, M., Burd, M., Adhikari, S., Sun, Y.-S., and Li, Q.-J. (2016). Coevolutionary elaboration of pollination-related traits in an alpine ginger (*Roscoea purpurea*) and a tabanid fly in the Nepalese Himalayas. *N. Phytol.* 211, 1402–1411. doi: 10.1111/nph.13974

Paudel, B. R., Shrestha, M., Dyer, A. G., Zhu, X.-F., Abdusalam, A., and Li, Q.-J. (2015). Out of Africa: evidence of the obligate mutualism between long corolla tubed plant and long-tongued fly in the Himalayas. *Ecol. Evol.* 5, 5240–5251. doi: 10.1002/ece3.1784

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Pickering, C. M., and Stock, M. (2003). Insect colour preference compared to flower colours in the Australian Alps. *Nordic J. Bot.* 23, 217–223. doi: 10.1111/j.1756-1051.2003.tb00384.x

Raine, N. E., and Chittka, L. (2005). Colour preferences in relation to the foraging performance and fitness of the bumblebee *Bombus terrestris*. *Uludag Bee J.* 5, 145–150.

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Revell, L. J., Harmon, L. J., and Collar, D. C. (2008). Phylogenetic signal, evolutionary process, and rate. *Syst. Biol.* 57, 591–601. doi: 10.1080/10635150802302427

Sargent, R. D., and Ackerly, D. D. (2008). Plant–pollinator interactions and the assembly of plant communities. *Trends Ecol. Evol.* 23, 123–130. doi: 10.1016/j.tree.2007.11.003

Schemske, D. W., Mittelbach, G. G., Cornell, H. V., Sobel, J. M., and Roy, K. (2009). Is there a latitudinal gradient in the importance of biotic interactions? *Annu. Rev. Ecol. Evol. Syst.* 40, 245–269. doi: 10.1146/annurev.ecolsys.39.110707.173430

Shrestha, M., Burd, M., Garcia, J. E., Dorin, A., and Dyer, A. G. (2019a). Colour evolution within orchids depends on whether the pollinator is a bee or a fly. *Plant Biol.* 21, 745–752. doi: 10.1111/plb.12968

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019b). Floral colour structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Garcia, J. E., Bukovac, Z., Dorin, A., and Dyer, A. G. (2018). Pollination in a new climate: assessing the potential influence of flower

temperature variation on insect pollinator behaviour. *PLoS One* 13:e0200549. doi: 10.1371/journal.pone.0200549

Shrestha, M., Garcia, J. E., Burd, M., and Dyer, A. G. (2020). Australian native flower colours: does nectar reward drive bee pollinator flower preferences? *PLoS One* 15:e0226469. doi: 10.1371/journal.pone.0226469

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Soltis, D. E., Smith, S. A., Cellinese, N., Wurdack, K. J., Tank, D. C., Brockington, S. F., et al. (2011). Angiosperm phylogeny: 17 genes, 640 taxa. *Am. J. Bot.* 98, 704–730.

Stournaras, K. E., Lo, E., Böhning-Gaese, K., Cazetta, E., Dehling, D. M., Schleuning, M., et al. (2013). How colorful are fruits? Limited color diversity in fleshy fruits on local and global scales. *N. Phytol.* 198, 617–629. doi: 10.1111/nph.12157

Tai, K.-C. (2018). *Pollinator Preference and Pollinator-Mediated Competition United to Shape the Floral Color Assembly Along Altitudes in Taiwan*. Master thesis. Taipei: National Taiwan University.

Tai, K.-C., Shrestha, M., Dyer, A. G., Yang, E.-C., and Wang, C.-N. (2020). Data from: Floral colour diversity in the tropical-subtropical mountainous island of Taiwan. *Dryad Dig. Repos.* doi: 10.5061/dryad.63xsj3v08. [Epub ahead of print].

Thapa, V. K. (2000). *An Inventory of Nepal Insect. Volumn III (Hemiptera, Hymenoptera, Coleoptera & Diptera)*. Kathmandu: IUCN.

Totland, Ø (1993). Pollination in alpine Norway: flowering phenology, insect visitors, and visitation rates in two plant communities. *Can. J. Bot.* 71, 1072–1079. doi: 10.1139/b93-124

van der Kooi, C. J., Pen, I., Staal, M., Stavenga, D. G., and Elzenga, J. T. M. (2016). Competition for pollinators and intra-communal spectral dissimilarity of flowers. *Plant Biol.* 18, 56–62. doi: 10.1111/plb.12328

van Kleunen, M., Dawson, W., Essl, F., Pergl, J., Winter, M., Weber, E., et al. (2015). Global exchange and accumulation of non-native plants. *Nature* 525, 100–103. doi: 10.1038/nature14910

Voris, H. K. (2000). Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *J. Biogeogr.* 27, 1153–1167. doi: 10.1046/j.1365-2699.2000.00489.x

Warren, S. D., Harper, K. T., and Booth, G. M. (1988). Elevational distribution of insect pollinators. *Am. Midland Nat.* 120, 325–330. doi: 10.2307/2426004

Webb, C. J., and Kelly, D. (1993). The reproductive biology of the New Zealand flora. *Trends Ecol. Evol.* 8, 442–447. doi: 10.1016/0169-5347(93)90007-C

Webb, C. O., and Donoghue, M. J. (2005). Phylomatic: tree assembly for applied phylogenetics. *Mol. Ecol. Notes* 5, 181–183. doi: 10.1111/j.1471-8286.2004.00829.x

Weevers, T. (1952). Flower colours and their frequency. *Acta Bot. Neerland.* 1, 81–92. doi: 10.1111/j.1438-8677.1952.tb00002.x

Wikström, N., Kainulainen, K., Razafimandimbison, S. G., Smedmark, J. E. E., and Bremer, B. (2015). A revised time tree of the asterids: establishing a temporal framework for evolutionary studies of the coffee family (Rubiaceae). *PLoS One* 10:e0126690. doi: 10.1371/journal.pone.0126690

Wikström, N., Savolainen, V., and Chase, M. W. (2001). Evolution of the angiosperms: calibrating the family tree. *Proc. R. Soc. B* 268, 2211–2220. doi: 10.1098/rspb.2001.1782

Williams, P. H., Ito, M., Matsumura, T., and Kudo, I. (2010). The bumblebees of the Nepal Himalaya (Hymenoptera : Apidae). *Insecta Matsumurana* 66, 115–151.

Wilson, M. F., and von Neumann, R. A. (1972). Why are Neotropical birds more colourful than North American birds? *Avicult. Magaz.* 78, 141–147.

Wu, F. T. (1978). Recent tectonics of taiwan. *J. Phys. Earth* 26, S265–S299. doi: 10.4294/jpe1952.26.Supplement_S265

Zanne, A. E., Tank, D. C., Cornwell, W. K., Eastman, J. M., Smith, S. A., FitzJohn, R. G., et al. (2014). Three keys to the radiation of angiosperms into freezing environments. *Nature* 506, 89–92. doi: 10.1038/nature12872

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Erratum: Floral Color Diversity: How Are Signals Shaped by Elevational Gradient on the Tropical–Subtropical Mountainous Island of Taiwan?

*Frontiers Production Office**

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Due to a production error, there was a mistake in **Table 2** as published. The heading mistakenly contained "B =" which should have been removed. The corrected heading and **Table 2** appear below.

The publisher apologizes for this mistake. The original article has been updated.

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TABLE 2 | Summary of the phylogenetic structure of low, middle and high-altitude species in Taiwan.

	Altitude	*N*	Actual	Nulls (mean ± sd)	*P*
MPD_{phylo}					
	Low	399	268.1	267.0 ± 3.0	0.639
	Middle	186	267.8	267.1 ± 5.7	0.54
	High	142	260.6	267.0 ± 6.8	0.174
$MNTD_{phylo}$					
	Low	399	94	93.3 ± 2.1	0.645
	Middle	186	106.4	111.8 ± 4.2	0.098
	High	142	105.3	118.9 ± 5.0	0.004

The P-value is determined by the rank of the actual MPD_{phylo} (or $MNTD_{phylo}$) in 1000 nulls MPD_{phylo} (or $MNTD_{phylo}$). Larger P-value indicates stronger phylogenetic over-dispersion, while lower P-value indicates stronger phylogenetic cluster. Phylogenetic distance calculated from the phylogenetic tree was the distance analyzed here.

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ORIGINAL RESEARCH
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Indirect Selection on Flower Color in *Silene littorea*

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Flower color, as other floral traits, may suffer conflicting selective pressures mediated by both mutualists and antagonists. The maintenance of intraspecific flower color variability has been usually explained as a result of direct selection by biotic agents. However, flower color might also be under indirect selection through correlated traits, since correlations among flower traits are frequent. In this study, we aimed to find out how flower color variability is maintained in two nearby populations of *Silene littorea* that consistently differ in the proportions of white-flowered plants. To do that, we assessed natural selection on floral color and correlated traits by means of phenotypic selection analysis and path analysis. Strong directional selection on floral display and flower production was found in both populations through either male or female fitness. Flower color had a negative indirect effect on the total male and female fitness in Melide population, as plants with lighter corollas produced more flowers. In contrast, in Barra population, plants with darker corollas produced more flowers and have darker calices, which in turn were selected. Our results suggest that the prevalence of white-flowered plants in Melide and pink-flowered plants in Barra is a result of indirect selection through correlated flower traits and not a result of direct selection of either pollinators or herbivores on color.

Keywords: calyx color, corolla color, floral display, florivory, male and female fitness, path analysis, phenotypic selection

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INTRODUCTION

Biotic and abiotic agents of selection may affect floral traits and, thereby, plants fitness. Among the biotic agents, pollinators have been considered the most important (Darwin, 1862; Schiestl and Johnson, 2013; Souto-Vilarós et al., 2018; Ramos and Schiestl, 2019) because, given their primary role in transferring pollen between plants (Barrett and Harder, 1992), they have a strong and direct impact on plant fitness (Waser and Price, 1981; Waser et al., 1996; Schemske and Bradshaw, 1999). However, some floral traits can be signals not only to pollinators but also to herbivores (e.g., Raguso, 2008) that directly or indirectly damage reproductive tissues, reducing plant reproductive success (Gómez, 2003; Irwin et al., 2003). In this way, the potential selection exerted by pollinators could be altered by herbivores, changing the strength and the direction of that selection (Strauss, 1997; Hambäck, 2001; Herrera et al., 2002; Irwin et al., 2003; Irwin and Strauss, 2005; Ågren, 2019; Ramos and Schiestl, 2019). Thus, some floral traits suffer conflicting selective pressures mediated by both mutualists and antagonists (but

see Veiga et al., 2015). In addition, the intensity of different biotic and abiotic interactions varies spatially, resulting in divergent selection and maintaining the variability of floral traits that influence these interactions (Mitchell-Olds et al., 2007; Ågren et al., 2013; Vaidya et al., 2018). The variability of floral traits may also result from indirect selection, imposed by either biotic or abiotic agents, on correlated vegetative traits (Simms and Bucher, 1996; Strauss and Whittall, 2006).

Floral color is a prevalent feature in the study of pollinator-mediated selection as it can offer a direct visual information to the flower visitors about the availability of rewards, and its variation at different levels (population, plant, flower) provides a basis for natural selection to occur (Kantsa et al., 2017; Wester and Lunau, 2017). Some pollinators can learn to associate specific flower colors with rewards, visiting preferentially those flowers (Grüter and Ratnieks, 2011; Grüter et al., 2011), which could cause directional selection on flower color. In fact, numerous studies have highlighted the role of pollinators in the evolution and maintenance of flower color diversity in plant clades (Kay and Sargent, 2009; van der Niet and Johnson, 2012), some of them in flower-color polymorphic species (Malerba and Nattero, 2012; Kellenberger et al., 2019). Although petal color may be affected by structural features of petal cells (Glover and Whitney, 2010; Airoldi et al., 2019; van der Kooi et al., 2019), it mainly results from the accumulation of pigments (Davies, 2000; Tanaka et al., 2008). Flavonoids are the principal source of flower color (Tanaka et al., 2008; Narbona et al., 2018), and they are related not only to pollinator attraction but also to defense against herbivory or pathogens or to protection from environmental stressors, such as UV-radiation, drought, temperature, or salinity (Chalker-Scott, 1999; Winkel-Shirley, 2002; Manetas, 2006; Gould et al., 2009; Lev-Yadun and Gould, 2009; Qi et al., 2011; Schaefer and Ruxton, 2011; del Valle et al., 2015; Chin et al., 2016; Davies et al., 2018). Therefore, flower color can act as a signal for mutualists and antagonists (Fineblum and Rausher, 1995; Irwin et al., 2003; Caruso et al., 2010; Ramos and Schiestl, 2019). In fact, some floral herbivores, such as Hemiptera (Farnier et al., 2014), Thysanoptera (Vernon and Gillespie, 1990; Gaum et al., 1994; Chyzik et al., 1995), Lepidoptera (Johnson et al., 2008), or pollen-feeding beetles (Giamoustaris and Mithen, 1996), discriminate among flower color variants resulting in higher attack and damage levels on some colors over others. Furthermore, it has been found that larvae from Lepidoptera species prefer to feed on flowers with low anthocyanin contents (Johnson et al., 2008) as these pigments reduce their growth (Johnson and Dowd, 2003; Johnson et al., 2008). Besides, due to those multiple roles of flavonoids, floral color may have diverse pleiotropic effects in other plant organs, through which selection, exerted by either biotic or abiotic factors, can indirectly act on flower color (Strauss and Whittall, 2006; Narbona et al., 2018).

Flower traits are frequently correlated (Mazer and Hultgård, 1993; Ishii and Morinaga, 2005). Diverse floral traits, such as floral display in *Lobularia* (Gómez, 2000), inflorescence production in *Hydrophyllum* (Wolfe, 1993), stigma-anther separation in *Ipomoea* (Sobrevila et al., 1989) and *Lysimachia* (Jiménez-López et al., 2020), nectar production in *Ipomopsis* (Meléndez-Ackerman and Campbell, 1998), or flower size in diverse crucifer species (Dukas and Shmida, 1989), have shown to be correlated to flower color. Given that biotic selective agents, such as pollinators and herbivores, also respond to other features apart from flower color, it is necessary to consider those correlations when studying selection on flower color (Kingsolver and Diamond, 2011).

Floral color has been mostly viewed by evolutionary biologists as a qualitative trait (Harder and Johnson, 2009), and studies assessing selection on that floral feature as a continuous trait are not frequent (but see Renoult et al., 2013; Sletvold et al., 2016). In shore campion (*Silene littorea* Brot.), flower color shows a genetically determined continuous variation from dark pink to white, which so far has been analyzed considering the dark pink, light pink, and white categories for simplicity, with frequency distribution of colors differing drastically among populations (Casimiro-Soriguer et al., 2016; Del Valle et al., 2019). Although *S. littorea* is self-compatible, it needs pollinator visitation to achieve complete seed production per fruit (Casimiro-Soriguer et al., 2015; Buide et al., 2020), and pollinators can thus be agents of phenotypic selection on flower color. Bees and butterflies were the main pollinators of *S. littorea* and they preferentially visited pink vs. white flowers (Buide et al., 2020). Since some pollinators, such as bees, can differentiate among flowers that differ subtly in color (Garcia et al., 2017), it is worth considering the whole variation from pink to white and the effect of such color variation on fitness. In addition, this species showed among-year variation in florivory, and white-flowered plants showed higher total levels of florivory (Buide et al., 2020). Moreover, the genus *Silene* usually exhibits a close relationship with the genus *Hadena*, which oviposits in ovaries and the larvae feed on the seeds (e.g. Koch, 1984; Giménez-Benavides et al., 2007; Kula et al., 2013); and in particular, *Hadena sancta* was found in *S. littorea* (Prieto-Benítez et al., 2017). In *S. littorea*, pigments responsible for pink petal coloration are anthocyanins, specifically cyanidin derivatives, which may also accumulate in sepals (Del Valle et al., 2019); their concentrations in corolla and calyx show wide variation and do not usually correlate (del Valle et al., 2015). The pink intensity of petals depends on their anthocyanin concentration (Casimiro-Soriguer et al., 2016); likewise, calyx color varies from green to dark red depending on anthocyanin accumulation (del Valle et al., 2015). That variability in pigment concentration, and thus in color, in petals and calyces could be involved in both pollinator and herbivore preferences. Hence, *S. littorea*, as a study model, gives an excellent opportunity to assess spatial variation in selection regimes on quantitative variation of floral color.

Here, we assess natural selection on the quantitative variation in the flower color found in two nearby populations of *S. littorea*, taking into account correlations to other floral traits and herbivore pressures. We use selection differentials and gradients to quantify phenotypic selection on floral traits through both male and female fitness, and we also use structural equation modeling (SEM) to disentangle the effects of floral traits and herbivory on female fitness and thus to unravel the ecological causes of selection. The simultaneous use of those analyses is

recommended when assessing selection on quantitative traits and dealing with multiple causal scenarios (Conner et al., 1996; Gómez, 2000; Scheiner et al., 2000). Specifically, we raise the following questions: (1) Does natural selection operate on continuous variation of floral color? (2) Does selection on floral color act directly or indirectly through other floral traits? (3) Do herbivores affect selection on flower color? and (4) Is there any variation in selection regime between populations?

MATERIALS AND METHODS

Study System

Silene littorea (Caryophyllaceae) is an annual plant distributed throughout the coast of the Iberian Peninsula and in the Northwest of Morocco (Talavera, 1990). It blooms between March and June and produces from only three to nearly 300 flowers per plant (Casimiro-Soriguer et al., 2013). It is a gynodioecious-gynomonoecious species with three sexual phenotypes: female plants, hermaphrodite plants, and gynomonoecious plants, with both female and hermaphrodite flowers (Guitián and Medrano, 2000; Casimiro-Soriguer et al., 2013). The relative frequencies of those sexual phenotypes are very variable among populations of Spain, female plants being scarce or absent in all reported populations and hermaphrodite plants being more frequent in populations of Northwest Spain (Guitián and Medrano, 2000; Casimiro-Soriguer et al., 2013). Hermaphrodite flowers are protandrous with 10 stamens that open sequentially mostly before the receptivity of the stigma, but some overlaps between sexual phases exist (Casimiro-Soriguer et al., 2013). *Silene littorea* is a self-compatible species, with incomplete protandry that allows some levels of autonomous selfing (Casimiro-Soriguer et al., 2015; Buide et al., 2020). Most populations of *S. littorea* consist exclusively of plants with pink flowers, while a few populations in the northwest of Spain also include white-flowered plants in proportions that differ across populations but are maintained over years (Casimiro-Soriguer et al., 2016; Del Valle et al., 2019). These populations include a continuous variation from dark-pink- to white-flowered plants, through a gradation of pinks (**Figure 1**). There is no information about the selective regimes operating to maintain flower color variability among plants in these populations.

This study was carried out during the spring season of 2016 in two wild populations in north-western Spain, Barra (42°15'42.20"N, 8°51'10.49"W) and Melide (42°15'5.18"N, 8°51'58.64"W), only separated by 2.6 km. Barra has maintained different proportions of white-flowered plants ranging from 14 to 20% through 3 consecutive years recorded (Del Valle et al., 2019). In the studied year, the proportions of white-flowered plants were 20% in Barra and 65% in Melide.

Floral Traits

In this study, we have included those floral characters involved in attracting pollinators, such as flower color, flower size and floral display, on the one hand. Since some Lepidoptera oviposit in the ovary though the calyx and feed on the ovules and seeds of *Silene* (see above), we have considered, on the other hand, the color and size of the calyx and the number of ovules as factors that can affect the impact of herbivory. The number of flowers per plant was also included as it can have a direct relationship with the total fitness of the plant. Corolla size (measured as total area) and color, calyx length and color, ovules per flower, floral display, and total number of flowers per plant were recorded in 46–49 plants in Barra and 42–50 in Melide. Initially, 50 plants

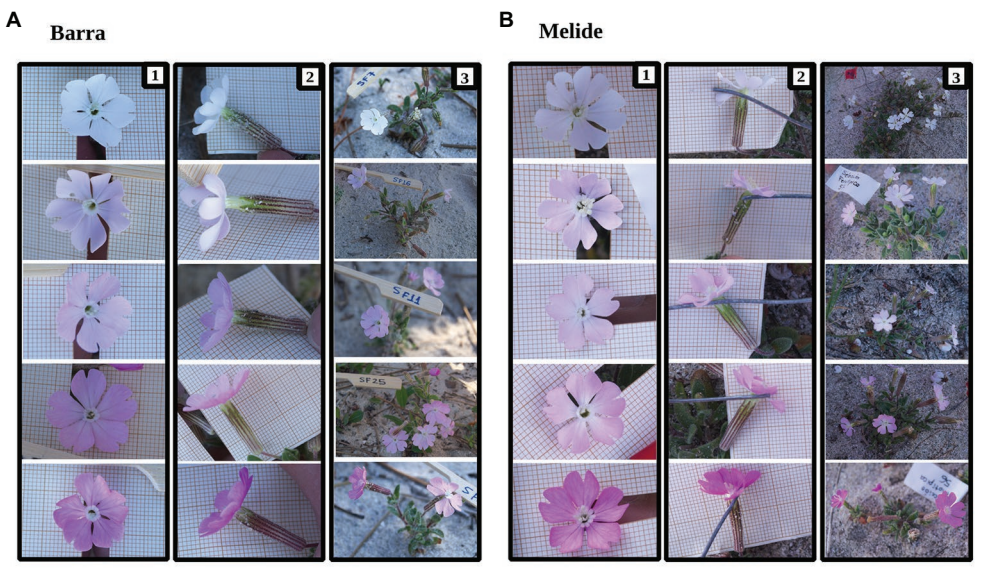


FIGURE 1 | Plants of *Silene littorea* from Barra and Melide populations showing corolla color variation from dark pink to white (**A1**,**B1**), anthocyanin accumulation in calyces (i.e., redness; **A2**,**B2**), and the whole plant (**A3**,**B3**).

were tagged in each population, but in the end, there were some missing data for different traits. To avoid bias in phenotypic selection analyses due to different abundances of flower colors between populations, plants sampled in each population were evenly distributed along the corolla color gradient, from dark pink to white. In contrast, gender was not taken into account when sampling those plants, that is, plants were selected at random with respect to that feature although their gender expression was assessed when estimating flower production (see below). To measure color and size traits, a flower per plant was photographed in both lateral and zenith views with a digital camera (SONY SLT A65V) at the flowering peak; lateral pictures were used to measure calyx traits and zenith ones to characterize corolla traits. Each picture included a scale so that flower size was characterized, and the software ImageJ (version 1.52a, National Institutes of Health, USA) was used to measure corolla area and calyx length. Corolla and calyx colors were assessed on those pictures following the method described by Del Valle et al. (2018), which uses the output data from digital images to calculate indices that correlate to anthocyanin concentrations. Briefly, camera settings were manually adjusted for pictures: lens aperture F/5.6, ISO 100, white balance fixed at 4,500 K, and the integration time was changed depending on the specific daylight conditions (1/30 to 1/100). Pictures were taken in Sony Alpha Raw format (RAW) and flowers were photographed along with a Color-Checker Passport (X-Rite Inc., Grand Rapids, MI). Photographs were normalized and linearized using "Image Calibrations and Analysis Toolbox," a plug-in module for ImageJ (Troscianko and Stevens, 2015), using two gray standards of the Color-Checker: Neutral 3.5 and Neutral 8. From each image, we selected a specific area of either corolla or calyx to be analyzed and extracted its mean pixel values of the red (R), green (G), and blue (B) channels. Then, anthocyanin concentration was estimated by the R:G ratio, that is, the mean pixel value of the red channel divided by that of the green channel, which is one of the indices that better correlates to anthocyanin concentration and color based on reflectance spectra in petals and calyxes of *S. littorea* (del Valle et al., 2018).

For each plant, ripe seeds and aborted ovules were counted in three unopened fruits; then, the number of ovules per flower was obtained by adding ripe seeds and aborted ovules. The floral display of each plant was obtained by counting all open flowers 1 day at the peak of flowering. Lastly, the total number of flowers produced per plant was estimated by counting weekly all open flowers along the flowering period, each flower being marked to avoid recounting it, and female and hermaphrodite flowers being recorded separately.

Herbivory

Herbivory was assessed, as both corolla and ovary predation, for 49–50 of the plants used to measure floral traits, in each population. The percentage of corolla area predated was calculated on the same photographs used to measure the corolla size by using ImageJ software. For each plant, ovary predation was assessed by counting all ovaries showing chewing damage, and from that number, the proportion of predated ovaries, relative to total ovaries, was calculated.

Fitness Estimates

In each population, most sampled plants were hermaphrodites and only a few were gynomonoecious. In *S. littorea*, female fitness has been reported to be independent from flower gender (Guitián and Medrano, 2000; Casimiro-Soriguer et al., 2013). Thus, for gynomonoecious plants, both female and hermaphroditic flowers were used to estimate female fitness and only hermaphroditic flowers to assess male fitness. For hermaphrodite plants, all the flowers were used to estimate both female and male fitness.

Female fitness was assessed in each population for 42–50 of the plants used to measure floral traits. For each plant, fruit production was assessed by counting all fruits produced and the fruit-set (proportion of flowers setting fruits) was estimated by relating that the number of fruits to that of total flowers produced. To estimate seed production per fruit and seed-set (proportion of ovules becoming seeds), both ripe seeds and aborted ovules were counted in three unopened fruits per plant. Lastly, the total female fitness were estimated for each plant as its total seed production, that is, as the product of its mean number of ripe seeds per fruit with its total fruit production.

Male fitness was assessed for a subsample of the plants used to measure floral traits (20 plants in Barra and 22 in Melide). The number of dispersed pollen grains was used as a proxy for male fitness (Snow and Lewis, 1993; Holland et al., 2004; Arista and Ortiz, 2007), estimating the number of pollen grains that remains in the anther after pollination. From each plant, one or two anthers per flower were collected just before their abscission and fixed in 70% ethanol. To count the number of undispersed pollen grains, the remaining pollen of each anther was dispersed in 1.5 ml of soapy distilled water. For each sample, the exact number of pollen grains was counted in 10 subsamples of 10 μl under a microscope. From this mean, the total number of undispersed pollen grains was determined. Then, for each flower, the number of dispersed pollens was estimated by subtracting the undispersed pollen grains from the mean number of pollen grains produced per flower of each population. Pollen production per flower from these populations had been previously obtained (21,711.33 ± 620.28; $n = 30$ in Barra and 18,192.3 ± 561.4; $n = 42$ in Melide; $t = 4.21$, $df = 65.08$, $p < 0.00001$). Lastly, the total male fitness for each plant was estimated as the product of its number of pollen grains dispersed per flower by its number of hermaphrodite flowers.

Statistical Analysis

Data exploration was performed before analysis: normality was checked by the Shapiro-Wilk test using R package "Stats" (v3.6.2), and variance homogeneity was checked by the Levene test using R package "Car" (v3.0-8; Fox and Weisberg, 2019). Then, differences in floral traits, fitness, and herbivory between populations were tested using the Wilcoxon rank-sum test for all variables except for the calyx color index, the number of

ovules per plant, the seed-set, and the number of seed per fruit, the only parametric ones, where *t*-test was used; both tests were performed with R package "Stats" (v3.6.2). Pairwise relationships among corolla color, floral display, and the number of flowers per plant were tested through Pearson correlations using the R package "Psych" (v1.9.12.31; Revelle, 2020). Before analyses, all variables were centered for each population to a zero mean with R package "Caret" (v6.0-86; Kuhn et al., 2016).

To explore whether the studied floral traits were under selection, we estimated selection opportunities, differentials, and gradients (Lande and Arnold, 1983; Arnold and Wade, 1984a,b). All those coefficients were estimated through both total male and total female fitness and separately for each population. We used the number of pollen grains dispersed and the number of seeds produced per plant as absolute measures of total male and female fitness, respectively. In order to better understand selection regimes in each studied population, selection differentials and gradients were also estimated through each of the partial components of fitness considered. Before estimating selection coefficients, all fitness measures were relativized (absolute value of each plant divided by the mean value in the population). Moreover, to calculate selection coefficients, each floral trait was standardized for each population to a zero mean and unit variance. We calculated selection opportunities as the variances in either total male or total female relative fitness; those coefficients measure the constraint on the evolutionary response imposed by fitness variability (Crow, 1958; O'Donald, 1970; Wade, 1979; Wade and Arnold, 1980; Arnold and Wade, 1984a,b; Moorad and Wade, 2013). To check for differences in selection opportunities between populations, we used R package "Car" (v3.0-8; Fox and Weisberg, 2019) to perform the Brown-Forsythe test, which is a robust test to compare variances of non-parametric data. We used R package "pbdDMAT" (v0.5-1; Schmidt et al., 2012) to estimate standardized selection differential (S') for each trait as the covariance between the standardized trait and either total male or total female relative fitness or any of the partial components of fitness considered; this coefficient measures the total selection acting on each trait, including both direct selection on the focal trait and indirect selection though correlated traits (Lande and Arnold, 1983). Lastly, we used multiple regression analyses to estimate standardized selection gradients, which indicate the force and direction of selection acting directly on each trait (Lande and Arnold, 1983). We used R package "Stats" (v3.6.2) to perform linear regressions of either total male or total female relative fitness or any of the partial components of fitness considered on all standardized traits; partial-regression coefficients (β') are standardized linear selection gradients representing the magnitude and direction of directional selection (Lande and Arnold, 1983).

To investigate complex multivariate causal models of plant phenotypic traits and herbivores on total female fitness, we used SEM, specifically path analysis (Grace, 2006; Rosseel, 2012). This analysis technique is appropriate to test our hypothesis, since it allows the simultaneous evaluation of the effects of multiple pathways between variables by comparing observed and expected covariance matrices. Our hypothesis was tested in models using unstandardized data (Grace, 2006), except for total female fitness, which was divided by 1,000 to reduce the effect of a larger variance. Corolla color was defined as an independent variable, and calyx color, total number of flowers per plant, percentage of corolla area predated, proportion of ovaries predated per plant, and female fitness (estimated as total seeds produced per plant) were defined as dependent variables. To choose the model that best fit our data, we first used an alternative model evaluation approach (Hershberger et al., 2003; Grace, 2006), by comparing near-saturated models (i.e., models with only one and two degrees of freedom) differing in the order of unconnected variables (see the saturated model in **Supplementary Figure S1**). This process generated 102 different models for each population (**Supplementary Table S1**). We used this method because, *a priori*, all paths connecting the variables could be biologically possible and relevant (see for instance Cariveau et al., 2004; Swope and Parker, 2012; Carlson and Holsinger, 2013). The best-fitting model was chosen based on the lowest Akaike information criterion (AIC) values (Grace, 2006), which also were consistent with the Bayesian information criterion (BIC). Second, we performed the nested model comparisons using the best-fitting model as the baseline model (Grace, 2006). Model trimming was achieved by eliminating pathways with the lowest path coefficients, non-statistically supported. Comparisons between baseline model and reduced models were tested with Chi-square tests based on the differences of the two models' adjustment (Pugesek, 2003; Grace, 2006). However, differences between Chi-squares do not allow discrimination among the nested models (values of $p < 0.4$; **Supplementary Table S2**) and, thereby, we again applied the lowest AIC and BIC criteria.

Path analyses were performed using the "sem" function from R package "Lavaan" version 0.6-6 (Rosseel, 2012, 2020) with the maximum likelihood estimation of parameters. Because the data were not the multivariate normal distribution, we used bootstrap-based estimates of standard errors ($n = 1{,}000$; Grace, 2006; Rosseel, 2020). All statistical packages used throughout this article were implemented in R v.3.6.3 (R Core Team, 2020).

RESULTS

Variability in Floral Traits, Herbivory, and Fitness

As expected, the studied samples of both populations did not differ in corolla color (**Table 1**) because the previous selection of plants to include the overall color variation in both populations (see methods). Plants from Melide had smaller flowers with fewer ovules and darker calyces than those from Barra (**Table 1**). For all other traits, no differences were found between populations. Floral display and the total number of flowers per plant showed the highest variation among all the measured floral traits (**Table 1**), and these two traits were correlated in both populations (Barra: Pearson coefficient = 0.49, $p < 0.001$, $n = 74$; Melide:Pearson coefficient = 0.83, $p < 0.001$, $n = 50$).

The percentage of corolla area predated was similar in both populations. Likewise, no differences between populations were

TABLE 1 | Floral traits and herbivory and fitness estimates for Barra and Melide populations of *Silene littorea*.

	Barra					Melide					Between population comparisons
	Mean ± SD	Min	Max	N	CV %	Mean ± SD	Min	Max	N	CV %	
Floral traits											
Corolla color	1.29 ± 0.18	1.02	1.68	49	14.17	1.25 ± 0.17	1.03	1.60	50	13.23	$W = 1{,}371$, NS
Calyx color	1.35 ± 0.10	1.11	1.63	47	7.51	1.43 ± 0.10	1.17	1.69	50	7.18	$t = -3.716$, $p < 0.001$
Flower size (cm^2)	2.46 ± 0.50	1.38	3.93	49	20.38	1.85 ± 0.41	1.29	3.26	50	22.25	$W = 2{,}040$, $p < 0.0001$
Calyx length (cm)	1.71 ± 0.26	0.92	2.42	46	14.96	1.78 ± 0.16	1.44	2.25	50	9.24	$W = 973$, NS
Ovules per flower	66.85 ± 10.00	38.67	85.00	46	14.96	62.20 ± 8.07	40.00	77.33	42	12.98	$t = 2.4092$, $p < 0.05$
Floral display	2.51 ± 1.52	1.00	7.00	49	60.37	3.02 ± 2.22	1.00	13.00	50	73.43	$W = 1{,}088$, NS
Flowers per plant	17.41 ± 11.40	1.00	51.00	49	65.49	17.12 ± 12.44	2.00	53.00	50	72.67	$W = 12{,}81.5$, NS
Herbivory estimates											
Predated corolla area (%)	0.49 ± 1.04	0.00	5.52	49	213.82	1.00 ± 1.56	0.00	6.10	50	155.93	$W = 986.5$, NS
Ovaries predated per plant (%)	0.14 ± 0.15	0.00	0.62	49	105.31	0.18 ± 0.16	0.00	0.67	50	90.28	$W = 1{,}054.5$, NS
Ovaries predated per plant	2.71 ± 3.21	0.00	14.00	49	118.43	3.66 ± 4.70	0.00	24.00	50	128.34	$W = 1{,}101.5$, NS
Fitness estimates											
Fruit-set	0.32 ± 0.16	0.00	0.78	49	48.72	0.24 ± 0.15	0.00	0.75	50	62.99	$W = 1{,}643.5$, $p < 0.005$
Seed-set	0.65 ± 0.19	0.16	0.95	46	30.07	0.51 ± 0.22	0.03	0.91	42	44.21	$t = 3.1574$, $p < 0.005$
Fruits per plant	5.78 ± 4.33	0.00	19.00	49	75.00	3.86 ± 2.93	0.00	15.00	50	76.03	$W = 1{,}606.5$, $p < 0.01$
Seeds per fruit	43.04 ± 14.00	9.50	72.67	46	32.53	30.79 ± 14.99	2.00	64.33	43	48.69	$t = 3.9771$, $p < 0.0005$
Seeds per plant	272.69 ± 251.55	0.00	1,095.67	49	92.25	126.37 ± 139.11	0.00	710.00	47	110.08	$W = 1{,}609.5$, $p < 0.001$
Dispersed pollen grains per flower	17,856.10 ± 5,076.38	602.33	21,252.33	20	28.43	16,222.72 ± 3,206.86	4,833.45	18,258.45	22	19.77	$W = 331$, $p < 0.01$
Dispersed pollen grains per plant	427,694.97 ± 258,064.62	12,649.00	985,552.33	20	60.34	355,934.71 ± 251,876.43	64,633.81	919,202.98	22	70.76	W = 262, NS

Statistical comparisons between populations is showed. Corolla and calyx color index were measured as R:G ratio (see section "Materials and Methods").

found on number and percentage of predated ovaries (**Table 1**). No direct observation was made on the activity of predation agents, but *Hadena* larvae and Curculionidae eggs were found in flowers and fruits examined to characterize floral traits and to assess fitness.

All estimates of female fitness differed between populations. Fruit-set, seed-set, and seeds per fruit were markedly higher in Barra (**Table 1**), but the most marked difference affected seed production per plant, which was more than twice in Barra than in Melide, even though the total flowers per plant did not differ between populations (**Table 1**). In contrast, differences between populations in male fitness were much less marked, and although the two estimates considered showed a trend to be higher in Barra, only the difference in the number of pollen grains dispersed per flower was significant (**Table 1**).

Phenotypic Selection on Flower Color and Other Flower Traits

Selection opportunities did not differ between populations either for total female fitness (0.85 in Barra and 1.21 in Melide; $F_{1,94} = 0.06$, $p = 0.81$, Brown-Forsythe test) or total male fitness (0.36 in Barra and 0.50 in Melide; $F_{1,40} = 0.54$, $p = 0.47$, Brown-Forsythe test). In contrast, patterns of selection varied between the two populations (**Table 2**).

In Barra population, four traits were under both indirect (S' significant) and direct (β' significant) selection by one or more fitness measures, one trait was only under indirect selection, and another one was only under direct selection (**Table 2**). Corolla color was under indirect positive selection *via* dispersed grains/plant and under direct negative selection *via* seed-set and seeds/fruit. Calyx color was under indirect positive selection *via* fruits/plant, seeds/plant and dispersed grains/plant and under direct positive selection *via* fruits/plant. Corolla size was under direct negative selection *via* seeds/fruit. The number of ovules per flower was under indirect positive selection *via* fruits/plant, seeds/fruit, seeds/plant, and dispersed grains per plant and under direct positive selection *via* seeds/fruit. Floral display was under indirect positive selection *via* fruits/plant, seeds/plant, and dispersed grains/plant. Flowers per plant was under indirect positive selection *via* fruits/plant, seeds/fruit, seeds/plant, and dispersed grains/plant and under direct positive selection *via* fruits/plant, seed-set, seeds/fruit, seeds/plant, and dispersed grains/plant.

In Melide population, two traits were under both indirect and direct selection, three traits were only under indirect selection and one was only under direct selection (**Table 2**). Corolla color was under indirect negative selection *via* fruits/plant, seeds/plant and dispersed grains/plant; and under indirect positive selection *via* dispersed grains/flower. Calyx color was under indirect negative selection *via* dispersed grains/plant. Corolla size was under direct negative selection *via* dispersed grains/plant. Number of ovules per flower was under indirect positive selection *via* dispersed grains/plant. Floral display was under indirect positive selection *via* fruits/plant, seeds/plant and dispersed grains/plant; and under direct positive selection *via* dispersed grains/plant. Flowers per plant were under indirect and direct positive selection *via* fruits/plant, seeds/plant and dispersed grains/plant.

Effects of Floral Traits and Herbivores on Total Female Fitness

After the alternative model evaluation and subsequent nested model comparisons (**Supplementary Table S1**), the models with the best fit for Barra and Melide populations are shown in **Figure 2**. In Barra, the model retains five significant casual pathways, one non-significant casual path and two unresolved causal relationships, whereas in Melide the model retains three significant casual pathways, five non-significant casual pathways and one unresolved causal relationships (**Supplementary Table S2**; **Figure 2**).

According to the model selected for Barra, the number of flowers per plant had the highest significant positive effect (i.e., path coefficient) on total female fitness (seeds per plant; standardized path coefficient = 1.03, **Figure 2A**). The only significant negative effect was the number of predated ovaries per plant on total female fitness (standardized path coefficient = −0.45). In addition, the number of predated ovaries was positively affected by flower number (standardized path coefficients = 0.58), and both calyx color and flower number were positively influenced by corolla color (standardized path coefficients = 0.36 and 0.30, respectively). As a result, corolla color showed a significant positive indirect effect on female fitness *via* the number of flowers (Estimate = 0.418, Z = 2.28, $p = 0.022$; **Supplementary Table S2**; **Figure 2A**).

In Melide, the number of flowers per plant was also the trait with the highest significant effect on female fitness, but the number of flowers also had important influence on ovary predation (standardized path coefficients = 0.82 and 0.76, respectively, **Figure 2B**). In this population, calyx color was not significantly affected by corolla color and female fitness was not significantly affected by predated ovaries per flower. Corolla color showed a significant negative effect on flower number (standardized path coefficient = −0.37), meaning that plants with lighter corollas had a higher number of flowers per plant. In that way, corolla color had a marginally significant negative indirect effect on female fitness *via* number of flower (Estimate = −0.257, $Z = -1.85$, $p = 0.064$; **Supplementary Table S2**; **Figure 2B**).

DISCUSSION

In this research, we found among-population differences in floral traits and in patterns of phenotypic selection in two nearby populations of *S. littorea* with a wide continuous variation in petal color from dark pink to white. Patterns of phenotypic selection varied through either male or female fitness although two traits were subjected to consistent and marked selective pressures in both populations: floral display and total number of flowers per plant.

In both populations, the number of flowers per plant was subjected to both direct and indirect selection through both total male and total female fitness, and path analysis confirmed

TABLE 2 | Phenotypic selection coefficients of floral traits of *S. littorea* through partial components of fitness, either female (fruit-set, fruits per plant, seed-set, and seeds per fruit) or male (dispersed pollen grains per flower), and through total female (seeds per plant) and total male (dispersed pollen grains per plant) fitness in Barra and Melide populations.

Population	Trait	Fruit-set		Fruits/plant		Seed-set		Seeds/fruit		Seeds/plant		Dispersed grains/flower		Dispersed grains/plant	
		S'	β'(SE)	S'	β'(SE)	S'	β'(SE)	S'	β'(SE)	S'	β'(SE)	S'	β'(SE)	S'	β'(SE)
Barra	Corolla color	−0.04	−0.07 (0.08)	0.16	−0.04 (0.07)	−0.06	**−0.11w*** (0.05)	−0.05	**−0.11*** (0.05)	0.15	−0.11 (0.1)	0.00	0 (0.12)	**0.19***	0.01 (0.1)
	Calyx color	0.02	0.08 (0.07)	**0.26****	**0.15*** (0.07)	−0.02	−0.01 (0.05)	0.00	0 (0.05)	**0.31****	0.19 (0.1)	0.07	0.08 (0.11)	**0.30*****	0.08 (0.09)
	Corolla size (cm^2)	−0.06	−0.08 (0.07)	0.03	−0.06 (−0.07)	−0.05	−0.09 (0.05)	−0.05	**−0.10*** (0.05)	0.00	−0.14 (0.09)	0.04	0.02 (0.1)	0.12	0 (0.08)
	Calyx length (cm)	0.12	0.08 (0.07)	0.06	0.07 (0.07)	0.00	−0.03 (0.05)	−0.02	−0.03 (0.05)	0.07	0.07 (0.09)	0.04	0.02 (0.11)	−0.05	0.05 (0.1)
	Ovules per flower	0.03	0.1 (0.09)	**0.34*****	0.11 (0.09)	−0.04	−0.04 (0.06)	**0.12****	**0.12*** (0.06)	**0.42*****	0.21 (0.12)	0.06	0.09 (0.15)	**0.26*****	0.06 (0.13)
	Floral display	0.02	0 (0.1)	**0.48*****	0.02 (0.1)	−0.02	−0.1 (0.07)	0.07	−0.1 (0.07)	**0.54*****	−0.09 (0.13)	0.02	−0.07 (0.13)	**0.38*****	−0.04 (0.11)
	Flowers per plant	0.04	−0.11 (0.1)	**0.62*****	**0.41***** (0.1)	0.03	**0.15*** (0.07)	**0.11***	**0.15*** (−0.07)	**0.71*****	**0.51***** (0.13)	0.04	−0.02 (0.13)	**0.56*****	**0.44**** (0.11)
Melide	Corolla color	−0.01	0 (0.11)	**−0.24***	−0.02 (0.1)	−0.01	−0.02 (0.09)	−0.02	−0.01 (0.09)	**−0.33***	−0.1 (0.17)	**0.06***	0.15 (0.07)	**−0.22***	0.15 (0.1)
	Calyx color	0.02	0.04 (0.1)	−0.06	−0.03 (0.09)	−0.02	−0.03 (0.08)	−0.01	−0.02 (0.08)	−0.06	−0.04 (0.14)	−0.03	0.01 (0.06)	−0.19*	−0.04 (0.08)
	Corolla size (cm^2)	−0.07	−0.15 (0.11)	0.03	−0.08 (0.1)	0.05	0.07 (0.09)	0.08	0.06 (0.09)	0.07	−0.04 (0.16)	−0.02	−0.13 (0.07)	0.00	−0.26* (0.1)
	Calyx length (cm)	0.10	0.08 (0.11)	−0.06	0 (0.1)	−0.04	−0.06 (0.09)	0.02	−0.06 (0.09)	−0.03	0.03 (0.17)	−0.03	0.08 (0.07)	−0.20	0.12 (0.1)
	Ovules per flower	−0.13	−0.09 (0.11)	0.11	−0.07 (0.1)	−0.01	0 (0.09)	0.12	0.14 (0.09)	0.23	−0.01 (0.16)	−0.01	−0.11 (0.08)	**0.24****	−0.14 (0.1)
	Floral display	−0.11	0.14 (−0.2)	**0.44*****	−0.05 (0.18)	0.02	−0.07 (0.16)	0.06	−0.07 (0.16)	**0.56*****	−0.28 (0.3)	0.02	0.3 (0.14)	**0.72*****	**0.53*** (0.19)
	Flowers per plant	−0.11	−0.26 (0.17)	**0.58*****	**0.61***** (0.15)	0.02	0.05 (0.14)	0.09	0.06 (0.14)	**0.78*****	**0.93***** (0.26)	−0.03	−0.1 (0.1)	**0.74*****	**0.41*** (0.13)

S', standardized selection differentials; β', standardized linear gradients of selection.

**Values in bold are significant: $p < 0.05$.*

***Values in bold are significant: $p < 0.01$.*

****Values in bold are significant: $p < 0.001$.*

a direct effect of floral production on total female fitness. The association between flower production and female fitness is quite common, and it has been found in a wide variety of plants (e.g. Herrera, 1993; Gómez, 2000; Harder and Johnson, 2009); however, the impact of flower production on male fitness has been more rarely reported (but see Maad, 2000). Total flower production and floral display showed a strong correlation in both populations, a fact that is usual (Worley and Barrett, 2000; Sletvold et al., 2016), although floral display may be more constant among species than the total flower number (Kudo and Harder, 2005). Floral display is one of the most important determinants of a plant's visual display and pollinators usually select plants with large floral displays (e.g., Eckhart, 1991; Thompson, 2001; Mitchell et al., 2004; Carlson and Holsinger, 2013). However, in our study, selection on floral display generally acted indirectly.

Since plants with large floral display and high flower production were strongly selected in both studied populations, an increase of these kinds of plants would be expected. However, these two traits showed the highest coefficients of variation among the studied traits, and in former studies, in these populations, flower production had also been reported to be highly variable (Casimiro-Soriguer et al., 2013). Other studies have reported a moderate heritability of flower production (Campbell and Halama, 1993; Worley and Barrett, 2000), and in *S. littorea*, this trait has a genetic component and also a plastic response to light conditions (Buide et al., 2018). These facts could be responsible for the maintenance of variability in this trait despite selective pressures against plants with fewer flowers. Herbivory could also be limiting the evolutionary response at least in Barra, since in both populations the path analysis revealed a positive effect of flower production on ovary predation that, in turn, had a negative effect on the total female fitness but that the latest effect was not significant in Melide.

Flower color showed different patterns of association with flower production in the two studied populations. In Melide, white- or lighter-flowered plants had higher production of flowers, and they were indirectly selected through both total male and total female fitness. In fact, in that population, path analysis revealed no direct effect of corolla color on total female fitness but an indirect effect through flower production, that is, lighter-flowered plants produced more flowers, and as a consequence, more seeds. The strong effect of flower production on total female fitness in that population could be hiding the impact of pollinators as selective agents on flower color. The lower fruit-set and seed-set recorded in that population could indicate a deficient pollinator visitation. However, there was no relation between flower color and either fruit-set or seed-set; and thus, plants in that population seem to receive low pollinator visits irrespective of their color, which would rule out pollinators as selective agents of flower color. Then, since predation of either flowers or ovaries had no effect on total female fitness in Melide, the overall results suggest that the high proportion of white-flowered plants in this population is a direct result of their higher fecundity due to their highest flower production.

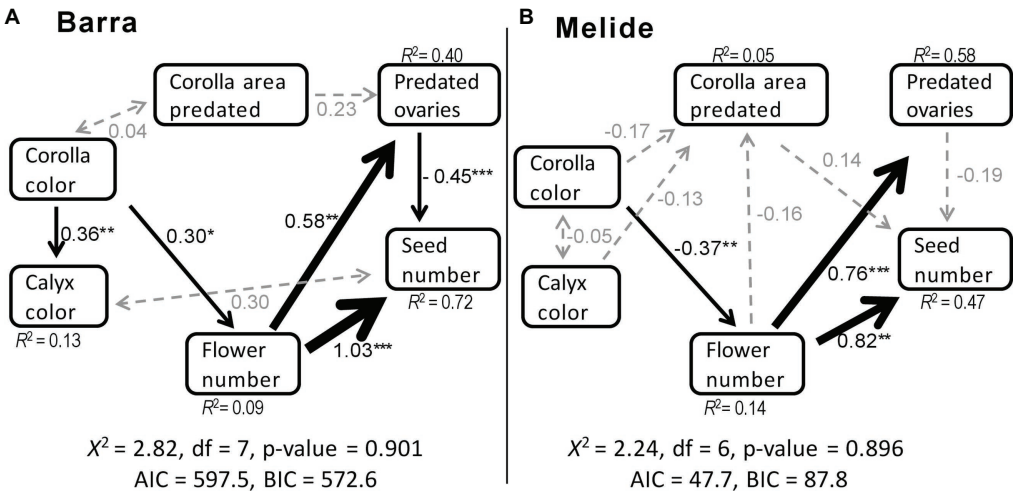


FIGURE 2 | Results of the best structural equation model for the effects of corolla color, calyx color, percentage of area of corolla predated, number of predated ovaries per flower, and number of flower per plant on seeds produced per plant in **(A)** Barra and **(B)** Melide populations of *S. littorea*. Black single-pointed arrows represent causal relationships with significant standardized path coefficients (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), gray single-pointed arrows with dashed lines represent causal relationships with non-significant path coefficients, and gray double-pointed arrows represent correlations. Arrow-width indicates the relative magnitude of the path coefficient. R^2 values are the percentage of variation explained by the causal variables. X^2, Chi-square test; AIC, Akaike information criterion; BCI, Bayesian information criterion.

A contrasting selective scenario appears in Barra, where indirect positive selection on petal color was found through absolute male fitness, that is, darker-flowered plants dispersed more pollen grains. Similar to that found in Melide, the path analysis does not show a direct effect of corolla color on total female fitness but an indirect effect through flower production. However, in Barra, the higher flower production was found in plants with darker flowers that consequently produced more seeds. Previous studies had found autonomous selfing in *S. littorea*, although pollinator visitation was required to reach full seed-set (Casimiro-Soriguer et al., 2015; Buide et al., 2020). In Barra population, Buide et al. (2020) reported that pollinators preferably visited pink flowers, and white-flowered plants under free pollination reached fruit-set values similar to those obtained by autonomous selfing. Thus, the lower male and female fitness of white-flowered plants in Barra could be related to their lower pollinator attraction, but the lower fitness seems mainly due to their lower flower production. However, their ability to produce fruits autonomously helps to compensate the scarcity of pollinators and to maintain corolla color variation in this population.

In Barra, ovary predation had a significant negative impact on total female fitness. In other species, such as *Petunia* (Johnson et al., 2008) and *Raphanus sativus* (Strauss et al., 2004), herbivory rates are higher in the flower parts or plants with lower levels of anthocyanins, and are thus less protected against herbivores. However, the path analysis does not support these hypotheses, as herbivory affected similarly all color phenotypes in the studied year. However, we cannot discard a different effect among years because in the previous year, we had found higher levels of florivory in white-flowered plants than in pink-flowered plants (Buide et al., 2020). Thus, future studies should include among-year variation.

Our results, although obtained with a limited sampling, suggest that the prevalence of white-flowered plants in Melide and pink-flowered plants in Barra can be an indirect effect of correlations between corolla color and other floral traits. In other species, when white-flowered mutants arise, they usually disappear or remain in low frequency in populations due to a variety of factors: low pollinator visitation, higher herbivory rates, poor abiotic performance, or genetic drift (Rausher, 2008; Narbona et al., 2018). Most populations of *S. littorea* through the Iberian Peninsula fit this situation as they have exclusively pink-flowered individuals and white mutants that sporadically appear are lost (Del Valle et al., 2019). The presence of white-flowered plants that are maintained over time occurs only in a few populations (Del Valle et al., 2019) and they dominate only in Melide. The strong correlation between flower production and petal color seems to explain the maintenance and high frequency of white- or pink- flowered plants in different populations and the soundness of the path-models support that result. The correlation between flower color and flower display or flower production has been found in other polymorphic species. In some taxa, such as *Lobularia maritima* (Gómez, 2000), *Claitonia virginica* (Frey, 2004), or *Protea aurea* (Carlson and Holsinger, 2013), the less pigmented morphs show higher floral display but in others, such as *Hydrophyllum appendiculatum* (Wolfe, 1993) or *Phlox drummondii* (Levin and Brack, 1995), the reverse situation is found. Those contrasting correlations between flower color and flower production can be explained by different pleiotropic relationships (Armbruster, 2002). However, in *S. littorea*, the correlation between petal color and flower production is not easy to explain, because it differed among populations.

Although abiotic conditions are seemingly similar in both populations, the fact that flowers in Melide are smaller and with fewer ovules and pollen grains could indicate a lower resource availability in that population. A possible explanation for the differences between the two populations could be an interactive effect between habitat characteristics; for instance, resource availability combined with biotic factors could result in the different correlations between flower number and color. In Barra, the higher pollinator visitation and lower florivory of pink flowers (Buide et al., 2020) could lead to a positive correlation between flower number and color, if one assumes that there is no resource limitation and that pink flowers are more costly due to pigment production (Drumm-Herrel and Mohr, 1985). In contrast, in Melide, the combination of resource limitation with a low prevalence of pollinators could result in the negative correlation between flower number and color, hence higher flower production in white-flowered plants. Anyway, a more thorough analysis of resources availability and biotic factors over years should be done in those populations to unravel that possible combined effect. Alternatively, between-population differences in floral correlations could also be explained as a founder effect (Forsman et al., 2012) or could be due to our limited sampling. Nevertheless, it is difficult to explain differences between populations without knowing the extent to which the relationship between flower color and number is genetically and/or environmentally controlled (Wennersten and Forsman, 2012).

The different patterns of selective regimes in the studied populations, if maintained over time, could have evolutionary consequences for the maintenance of color polymorphism in *S. littorea*. Given that this trait is genetically determined (Casimiro-Soriguer et al., 2016), an increase of white-flowered plants is expected in Melide while a decrease would be expected in Barra. Directional selection could lead to the loss of flower color polymorphism within populations, although inter-population variation in selection regime could maintain flower color variability in the species as well as autonomous selfing. Lastly, although flower color was not under direct selection by pollinators or herbivores in the studied year, among-year variation and other important traits as flower odor should be considered to support indirect selection on flower color in *S. littorea*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

EN and MB conceived the experiments. MB and NR-C carried out the sampling. NR-C performed the data analysis. NR-C, MA, EN, PO, and MB wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articless/10.3389/fpls.2020.588383/full#supplementary-material

REFERENCES

Ågren, J. (2019). Pollinators, herbivores, and the evolution of floral traits. *Science* 364, 122–123. doi: 10.1126/science.aax1656

Ågren, J., Hellström, F., Toräng, P., and Ehrlén, J. (2013). Mutualists and antagonists drive among-population variation in selection and evolution of floral display in a perennial herb. *Proc. Natl. Acad. Sci. U. S. A.* 110, 18202–18207. doi: 10.1073/pnas.1301421110

Airoldi, C. A., Ferria, J., and Glover, B. J. (2019). The cellular and genetic basis of structural colour in plants. *Curr. Opin. Plant Biol.* 47, 81–87. doi: 10.1016/j.pbi.2018.10.002

Arista, M., and Ortiz, P. L. (2007). Differential gender selection on floral size: an experimental approach using *Cistus salvifolius*. *J. Ecol.* 95, 973–982. doi: 10.1111/j.1365-2745.2007.01276.x

Armbruster, W. S. (2002). Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *J. Evol. Biol.* 15, 468–486. doi: 10.1046/j.1420-9101.2002.00399.x

Arnold, S. J., and Wade, M. J. (1984a). On the measurement of natural and sexual selection: theory. *Evolution* 38, 709–719. doi: 10.1111/j.1558-5646.1984.tb00344.x

Arnold, S. J., and Wade, M. J. (1984b). On the measurement of natural and sexual selection: applications. *Evolution* 38, 720–734. doi: 10.2307/2408384

Barrett, S. C. H., and Harder, L. D. (1992). Ecology and evolution of plant mating. *Trends Ecol. Evol.* 11, 73–79. doi: 10.1016/0169-5347(96)81046-9

Buide, M. L., del Valle, J. C., Castilla, A. R., and Narbona, E. (2018). Sex expression variation in response to shade in gynodioecious-gynomonoecious species: *Silene littorea* decreases flower production and increases female flower proportion. *Environ. Exp. Bot.* 146, 54–61. doi: 10.1016/j.envexpbot.2017.10.016

Buide, M. L., del Valle, J. C., Prado-Comesaña, A., and Narbona, E. (2020). The effects of pollination, herbivory and autonomous selfing on the maintenance

of flower colour variation in *Silene littorea*. *Plant Biol.* doi: 10.1111/plb.13209 [Epub ahead of print]

Campbell, D. R., and Halama, K. J. (1993). Resource and pollen limitations to lifetime seed production in a natural plant population. *Ecology* 74, 1043–1051. doi: 10.2307/1940474

Cariveau, D., Irwin, R. E., Brody, A. K., Garcia-Mayeya, L. S., and Von Der Ohe, A. (2004). Direct and indirect effects of pollinators and seed predators to selection on plant and floral traits. *Oikos* 104, 15–26. doi: 10.1111/j.0030-1299.2004.12641.x

Carlson, J. E., and Holsinger, K. E. (2013). Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171, 905–919. doi: 10.1007/s00442-012-2453-2

Caruso, C. M., Scott, S. L., Wray, J. C., and Walsh, C. A. (2010). Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *Int. J. Plant Sci.* 171, 1020–1028. doi: 10.1086/656511

Casimiro-Soriguer, I., Buide, M. L., and Narbona, E. (2013). The roles of female and hermaphroditic flowers in the gynodioecious-gynomonoecious *Silene littorea*: insights into the phenology of sex expression. *Plant Biol.* 15, 941–947. doi: 10.1111/j.1438-8677.2012.00697.x

Casimiro-Soriguer, I., Buide, M. L., and Narbona, E. (2015). Diversity of sexual systems within different lineages of the genus *Silene*. *AoB Plants* 7:plv037. doi: 10.1093/aobpla/plv037

Casimiro-Soriguer, I., Narbona, E., Buide, M. L., del Valle, J. C., and Whittall, J. B. (2016). Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (Caryophyllaceae). *Front. Plant Sci.* 7:204. doi: 10.3389/fpls.2016.00204

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol.* 70, 1–9. doi: 10.1111/j.1751-1097.1999.tb01944.x

Chin, H., Wu, Y., Hour, A., Hong, C., and Lin, Y. (2016). Genetic and evolutionary analysis of purple leaf sheath in rice. *Rice* 9, 1–14. doi: 10.1186/s12284-016-0080-y

Chyzik, R., Klein, M., Ben-Dov, Y., and Cohen, A. (1995). "Differential population density of Western flower thrips in various flower colors of gladiolus" in *Thrips biology and management*. eds. B. Parker, M. Skinner and T. Lewis (New York, USA: Plenum Press), 449–452.

Conner, J. K., Rush, S., and Jennetten, P. (1996). Measurements of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). I. Selection through lifetime female fitness. *Evolution* 50, 1127–1136. doi: 10.1111/j.1558-5646.1996.tb02353.x

Crow, J. F. (1958). Some possibilities for measuring selection intensities in man. *Hum. Biol.* 30, 1–13.

Darwin, C. R. (1862). *On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing*. London: John Murray.

Davies, K. M. (2000). "Plant colour and fragrance" in *Metabolic engineering of plant secondary metabolism*. eds. R. Verpoorte and A. W. Alfermann (Dordrecht: Springer).

Davies, K. M., Albert, N. W., Zhou, Y., and Schwinn, K. E. (2018). "Functions of flavonoid and betalain pigments in abiotic stress tolerance in plants" in *Annual Plant Reviews*. New York, NY, USA: John Wiley & sons, Ltd., 1–41.

Del Valle, J. C., Alcalde-Eon, C., Escribano-Bailón, M. T., Buide, M. L., Whittall, J. B., and Narbona, E. (2019). Stability of petal color polymorphism: the significance of anthocyanin accumulation in photosynthetic tissues. *BMC Plant Biol.* 19:496. doi: 10.1186/s12870-019-2082-6

Del Valle, J. C., Buide, M. L., Casimiro-Soriguer, I., Whittall, J. B., and Narbona, E. (2015). On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (*Silene littorea*). *Front. Plant Sci.* 6:939. doi: 10.3389/fpls.2015.00939

del Valle, J. C., Buide, M. L., Whittall, J. B., and Narbona, E. (2018). Phenotypic plasticity in light-induced flavonoids varies among tissues in *Silene littorea* (Caryophyllaceae). *Environ. Exp. Bot.* 153, 100–107. doi: 10.1016/j.envexpbot.2018.05.014

drumm-Herrel, H., and Mohr, H. (1985). Photosensitivity of seedlings differing in their potential to synthesize anthocyanin. *Physiol. Plant.* 64, 60–66. doi: 10.1111/j.1399-3054.1985.tb01213.x

Dukas, R., and Shmida, A. (1989). Correlation between the color, size and shape of Israeli crucifer flowers and relationship to pollinators. *Oikos* 54, 281–286. doi: 10.2307/3565286

Eckhart, V. M. (1991). The effects of floral display on pollinator visitation vary among populations of *Phacelia linearis* (Hydrophyllaceae). *Evol. Ecol.* 5, 370–384.

Farnier, K., Dyer, A. G., and Steinbauer, M. J. (2014). Related but not alike: not all Hemiptera are attracted to yellow. *Front. Ecol. Evol.* 2, 1–12. doi: 10.3389/fevo.2014.00067

Fineblum, W. L., and Rausher, M. D. (1995). Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature* 377, 517–520. doi: 10.1038/377517a0

Forsman, A., Wennersten, L., Karlsson, M., and Caesar, S. (2012). Variation in founder groups promotes establishment success in the wild. *Proc. R. Soc. B* 279, 2800–2806. doi: 10.1098/rspb.2012.0174

Fox, J., and Weisberg, S. (2019). *An R companion to applied regression*. *3rd Edn.* Thousand Oaks CA: Sage.

Frey, F. M. (2004). Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58, 2426–2437. doi: 10.1111/j.0014-3820.2004.tb00872.x

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path to colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Gaum, W. G., Giliomee, J. H., and Pringle, K. L. (1994). Resistance of some rose cultivars to the western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae). *Bull. Entomol. Res.* 84:487. doi: 10.1017/S0007485300032715

Giamoustaris, A., and Mithen, R. (1996). The effect of flower colour and glucosinolates on the interaction between oilseed rape and pollen beetles. *Entomol. Exp. Appl.* 80, 206–208.

Giménez-Benavides, L., Dötterl, S., Jürgens, A., Escudero, A., and Iriondo, J. M. (2007). Generalist diurnal pollination provides greater fitness in a plant with nocturnal pollination syndrome: assessing the effects of a *Silene-Hadena* interaction. *Oikos* 116, 1461–1472. doi: 10.1111/j.2007.0030-1299.15644.x

Glover, B. J., and Whitney, H. M. (2010). Structural colour and iridescence in plants: the poorly studied relations of pigment colour. *Ann. Bot.* 105, 505–511. doi: 10.1093/aob/mcq007

Gómez, J. M. (2000). Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *J. Evol. Biol.* 13, 689–699. doi: 10.1046/j.1420-9101.2000.00196.x

Gómez, J. M. (2003). Herbivory reduces the strength of pollinator-mediated selection in the mediterranean herb *Erysimum mediohispanicum*: consequences for plant specialization. *Am. Nat.* 162, 242–256. doi: 10.1086/376574

Gould, K. S., Davies, K. M., and Winefield, C. (2009). *Anthocyanins. Biosynthesis, functions and applications*. New York: Springer-Verlag.

Grace, J. B. (2006). *Structural equation modeling and natural systems*. New York, NY: Cambridge University Press.

Grüter, C., Moore, H., Firmin, N., Helanterä, H., and Ratnieks, F. L. (2011). Flower constancy in honey bee workers (*Apis mellifera*) depends on ecologically realistic rewards. *J. Exp. Biol.* 214, 1397–1402. doi: 10.1242/jeb.050583

Grüter, C., and Ratnieks, F. L. (2011). Flower constancy in insect pollinators: adaptive foraging behaviour or cognitive limitation? *Comm. Integr. Biol.* 4, 633–636. doi: 10.4161/cib.16972

Guitián, P., and Medrano, M. (2000). Sex expression and fruit set in *Silene littorea* (Caryophyllaceae): variation among populations. *Nord. J. Bot.* 20, 467–473. doi: 10.1111/j.1756-1051.2000.tb01589.x

Hambäck, P. (2001). Direct and indirect effects of herbivory: feeding by spittlebugs affects pollinator visitation rates and seedset of *Rudbeckia hirta*. *Ecoscience* 8, 45–50. doi: 10.1080/11956860.2001.11682629

Harder, L. D., and Johnson, S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence. *New Phytol.* 183, 530–545. doi: 10.1111/j.1469-8137.2009.02914.x

Herrera, C. M. (1993). Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecol. Monogr.* 63, 251–275. doi: 10.2307/2937101

Herrera, C. M., Medrano, M., Rey, P. J., Sánchez-Lafuente, A. M., García, M. B., Guitián, J., et al. (2002). Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16823–16828. doi: 10.1073/pnas.252362799

Hershberger, S. L., Marcoulides, G. A., and Parramore, M. M. (2003). "Structural equation modeling: an introduction" in *Structural equation modeling: Applications in ecological and evolutionary biology*. eds. B. H. Pugesek, A. Tomer and A. von Eye (Cambridge: Cambridge University Press), 3–41.

Holland, J. N., Bronstein, J. H., and DeAngelis, D. L. (2004). Testing hypotheses for excess flower production and low fruit-to-flower ratios in a pollinating seed-consuming mutualism. *Oikos* 105, 633–640. doi: 10.1111/j.0030-1299.2004.13058.x

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am. Nat.* 165, 225–237. doi: 10.1086/426714

Irwin, R. E., Strauss, S. Y., Storz, S., Emerson, A., and Guibert, G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84, 1733–1743. doi: 10.1890/0012-9658(2003)084[1733:TROHIT]2.0.CO;2

Ishii, H. S., and Morinaga, S. I. (2005). Intra- and inter-plant level correlations among floral traits in *Iris gracilipes* (Iridaceae). *Evol. Ecol.* 19, 435–448. doi: 10.1007/s10682-005-0896-1

Jiménez-López, F. J., Ortiz, P. L., Talavera, M., Pannell, J. R., and Arista, M. (2020). The role of lateral and vertical herkogamy in the divergence of the blue- and red-flowered lineages of *Lysimachia arvensis*. *Ann. Bot.* 125, 1127–1135. doi: 10.1093/aob/mcaa047

Johnson, E. T., Berhow, M. A., and Dowd, P. F. (2008). Colored and white sectors from star-patterned petunia flowers display differential resistance to corn earworm and cabbage looper larvae. *J. Chem. Ecol.* 34, 757–765. doi: 10.1007/s10886-008-9444-0

Johnson, E. T., and Dowd, P. F. (2003). "Plant anthocyanin insect feeding deterrents." in *Phytochemical Society of North America Annual Meeting Proceedings*, p. 8.

Kantsa, A., Raguso, R. A., Dyer, A. G., Sgardelis, S. P., Olesen, J. M., and Petanidou, T. (2017). Community-wide integration of floral colour and scent in a Mediterranean scrubland. *Nat. Ecol. Evol.* 1, 1502–1510. doi: 10.1038/s41559-017-0298-0

Kay, K. M., and Sargent, R. D. (2009). The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annu. Rev. Ecol. Syst.* 40, 637–656. doi: 10.1146/annurev.ecolsys.110308.120310

Kellenberger, R. T., Byers, K. J. R. P., Francisco, R. M. D., Staedler, Y. M., LaFountain, A. M., Schonenberger, J., et al. (2019). Emergence of a floral colour polymorphism by pollinator-mediated overdominance. *Nat. Commun.* 10:63. doi: 10.1038/s41467-018-07936-x

Kingsolver, J. G., and Diamond, S. E. (2011). Phenotypic selection in natural populations: what limits directional selection? *Am. Nat.* 177, 346–357. doi: 10.1086/658341

Koch, M. (1984). *Schmetterlinge*. Leipzig: Neumann.

Kudo, G., and Harder, L. D. (2005). Floral and inflorescence effects on variation in pollen removal and seed production among six legume species. *Funct. Ecol.* 19, 245–254. doi: 10.1111/j.1365-2435.2005.00961.x

Kuhn, M., Wing, J., Weston, S., Williams, A., Keefer, C., Engelhardt, A., et al. (2016). caret: Classification and Regression Training. R package version 6.0-71. Available at: https://CRAN.R-project.org/package=caret (Accessed November, 2020).

Kula, A. A., Dudash, M. R., and Fenster, C. B. (2013). Choices and consequences of oviposition by a pollinating seed predator, *Hadena ectypa* (Noctuidae), on its host plant, *Silene stellata* (Caryophyllaceae). *Am. J. Bot.* 100, 1148–1154. doi: 10.3732/ajb.1200636

Lande, R., and Arnold, S. J. (1983). The measurement of selection on correlated characters. *Evolution* 37, 1210–1226. doi: 10.1111/j.1558-5646.1983.tb00236.x

Levin, D. A., and Brack, E. T. (1995). Natural selection against white petals in *Phlox*. *Evolution* 49, 1017–1022. doi: 10.1111/j.1558-5646.1995.tb02336.x

Lev-Yadun, S., and Gould, K. S. (2009). "Role of anthocyanins in plant defence", *Anthocyanins: Biosynthesis, functions, and applications*. eds. K. S. Gould, K. M. Davies and C. Winefield (New York: Springer-Verlag).

Maad, J. (2000). Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution* 54, 112–123. doi: 10.1111/j.0014-3820.2000.tb00012.x

Malerba, R., and Nattero, J. (2012). Pollinator response to flower color polymorphism and floral display in a plant with a single-locus floral color polymorphism: consequences for plant reproduction. *Ecol. Res.* 27, 377–385. doi: 10.1007/s11284-011-0908-2

Manetas, Y. (2006). Why some leaves are anthocyanic and why most anthocyanic leaves are red? *Flora* 201, 163–177. doi: 10.1016/j.flora.2005.06.010

Mazer, S. J., and Hultgård, U. M. (1993). Variation and covariation among floral traits within and among four species of northern European *Primula* (Primulaceae). *Am. J. Bot.* 80, 474–485. doi: 10.1002/j.1537-2197.1993.tb13826.x

Meléndez-Ackerman, E., and Campbell, D. R. (1998). Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. *Evolution* 52, 1293–1303. doi: 10.1111/j.1558-5646.1998.tb02011.x

Mitchell, R. J., Karron, J. D., Holmquist, K. G., and Bell, J. M. (2004). The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Funct. Ecol.* 18, 116–124. doi: 10.1111/j.1365-2435.2004.00812.x

Mitchell-Olds, T., Willis, J. H., and Goldstein, D. B. (2007). Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nat. Rev. Genet.* 8, 845–856. doi: 10.1038/nrg2207

Moorad, J. A., and Wade, M. J. (2013). Selection gradients, the opportunity for selection, and the coefficient of determination. *Am. Nat.* 181, 291–300. doi: 10.1086/669158

Narbona, E., Wang, H., Ortiz, P., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the mediterranean basin: occurence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

O'Donald, P. (1970). Change of fitness by selection for a quantitative character. *Theor. Popul. Biol.* 1, 219–232. doi: 10.1016/0040-5809(70)90036-5

Prieto-Benítez, S., Yela, J. L., and Giménez-Benavides, L. (2017). Ten years of progress in the study o Hadena-Caryophyllaceae nursery pollination. A review in light of new Mediterranean data. *Flora* 232, 63–72. doi: 10.1016/j.flora.2017.02.004

Pugesek, B. H. (2003). "Modeling a complex conceptual theory of population change in the Shiras moose: history and recasting as a structural equation model" in *Structural equation modeling: Applications in ecological and evolutionary biology*. eds. B. H. Pugesek, A. Tomer and A. Von Eye Cambridge: Cambridge University Press, 60–84.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., et al. (2011). The jasmonate-ZIM-domain proteins interact with the WD-repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. *Plant Cell* 23, 1795–1814. doi: 10.1105/tpc.111.083261

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/ (Accessed November, 2020).

Raguso, R. A. (2008). The "invisible hand" of floral chemistry. *Science* 321, 1163–1164. doi: 10.1126/science.1163570

Ramos, S. E., and Schiestl, F. P. (2019). Rapid plant evolution driven by the interaction of pollination and herbivory. *Science* 364, 193–196. doi: 10.1126/science.aav6962

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Thomann, M., Schaefer, H. M., and Cheptou, P. O. (2013). Selection on quantitative colour variation in *Centaurea cyanus*: the role of the pollinator's visual system. *J. Evol. Biol.* 26, 2415–2427. doi: 10.1111/jeb.12234

Revelle, W. (2020). psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois; 2020, November. R package version 2.0.7. Available at: https://CRAN.R-project.org/package=psych.

Rosseel, Y. (2012). Lavaan: an R package for structural equation modeling. *J. Stat. Softw.* 48, 1–36. doi: 10.18637/jss.v048.i02

Rosseel, Y. (2020). The lavaan tutorial. Department of Data Analysis: Ghent University. Available at: https://lavaan.ugent.be/tutorial/tutorial.pdf (Accessed November, 2020).

Schaefer, H. M., and Ruxton, G. D. (2011). *Plant-animal comunication*. Oxford University Press.

Scheiner, S. M., Mitchell, R. J., and Callahan, H. S. (2000). Using path analysis to measure natural selection. *J. Evol. Biol.* 13, 423–433. doi: 10.1046/j.1420-9101.2000.00191.x

Schemske, D. W., and Bradshaw, H. D. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci. U. S. A.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28:307C315. doi: 10.1016/j.tree.2013.01.019

Schmidt, D., Chen, W.–C., Ostrouchov, G., and Patel, P. (2012). pbdDMAT: Distributed matrix algebra computation R package. Available at: http://cran.rproject.org/package=pbdDMAT (Accessed November 2020).

Simms, E., and Bucher, M. (1996). Pleiotropic effects of flower-color intensity on herbivore performance on *Ipomoea purpurea*. *Evolution* 50, 957–963. doi: 10.2307/2410871

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Snow, A. A., and Lewis, P. O. (1993). Reproductive traits and male fertility in plants: empirical approaches. *Annu. Rev. Ecol. Syst.* 24, 331–351. doi: 10.1146/annurev.es.24.110193.001555

Sobrevila, C., Wolfe, L. M., and Murcia, C. (1989). Flower polymorphism in the beach plant, *Ipomoea-imperati* (Vahl) Grisebach (Convolvulaceae). *Biotropica* 21, 84–88. doi: 10.2307/2388447

Souto-Vilarós, D., Vuleta, A., Jovanović, S. M., Budečević, S., Wang, H., Sapir, Y., et al. (2018). Are pollinators the agents of selection on flower colour and size in irises? *Oikos* 127, 834–846. doi: 10.1111/oik.04501

Strauss, S. Y. (1997). Floral characters link herbivores, pollinators, and plant fitness. *Ecology* 78, 1640–1645. doi: 10.1890/0012-9658(1997)078[1640:FCLHPA]2.0.CO;2

Strauss, S. Y., Irwin, R. E., and Lambrix, V. M. (2004). Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *J. Ecol.* 92, 132–141. doi: 10.1111/j.1365-2745.2004.00843.x

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits" in *Ecology and evolution of flowers*. eds. L. D. Harder and S. C. H. Barret (Oxford: Oxford University Press), 120–138.

Swope, S. M., and Parker, I. M. (2012). Complex interactions among biocontrol agents, pollinators, and an invasive weed: a structural equation modeling approach. *Ecol. Appl.* 22, 2122–2134. doi: 10.1890/12-0131.1

Talavera, S. (1990). "*Silene* L" in *Flora Iberica*. eds. S. Castroviejo, C. Aedo, M. Laínz, F. Muñoz Garmendia, G. Nieto Feliner and J. Paiva et al. (Madrid: Real Jardín Botánico).

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Thompson, J. D. (2001). How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system? *Oecologia* 126, 386–394. doi: 10.1007/s004420000531

Troscianko, J., and Stevens, M. (2015). Image calibration and analysis toolbox–a free software suite for objectively measuring reflectance, colour and pattern. *Methods Ecol. Evol.* 6, 1320–1331. doi: 10.1111/2041-210X.12439

Vaidya, P., McDurmon, A., Mattoon, E., Keefe, M., Carley, L., Lee, C. -R., et al. (2018). Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (*Boechera stricta*). *New Phytol.* 218, 380–392. doi: 10.1111/nph.14998

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Niet, T., and Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol. Evol.* 27, 353–361. doi: 10.1016/j.tree.2012.02.002

Veiga, T., Guitián, J., Guitián, P., Guitián, J., and Sobral, M. (2015). Are pollinators and seed predators selective agents on flower color in *Gentiana lutea*? *Evol. Ecol.* 29, 451–464. doi: 10.1007/s10682-014-9751-6

Vernon, R. S., and Gillespie, D. R. (1990). Spectral responsiveness of *Frankliniella-occidentalis* (Thysanoptera, Thripidae) determined by trap catches in greenhouses. *Environ. Entomol.* 19, 1229–1241. doi: 10.1093/ee/19.5.1229

Wade, M. J. (1979). Sexual selection and variance in reproductive success. *Am. Nat.* 114, 742–747. doi: 10.1086/283520

Wade, M. J., and Arnold, S. J. (1980). The intensity of sexual selection in relation to male sexual behaviour, female choice, and sperm precedence. *Anim. Behav.* 28, 446–461. doi: 10.1016/S0003-3472(80)80052-2

Waser, N. M., Chittka, L., Price, M. V., Williams, N., and Ollerton, J. (1996). Generalization in pollination systems, and why it matters. *Ecology* 77, 1043–1060. doi: 10.2307/2265575

Waser, N. M., and Price, M. V. (1981). Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* 35, 376–390. doi: 10.1111/j.1558-5646.1981.tb04896.x

Wennersten, L., and Forsman, A. (2012). Population-level consequences of polymorphism, plasticity and randomized phenotype switching: a review of predictions. *Biol. Rev.* 87, 756–767. doi: 10.1111/j.1469-185X.2012.00231.x

Wester, P., and Lunau, K. (2017). Plant-pollinator communication. *Adv. Bot. Res.* 82, 225–257. doi: 10.1016/bs.abr.2016.10.004

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.* 5, 218–223. doi: 10.1016/S1369-5266(02)00256-X

Wolfe, L. M. (1993). Reproductive consequences of a flower color polymorphism in *Hydrophyllum appendiculatum*. *Am. Midl. Nat.* 129, 405–408. doi: 10.2307/2426522

Worley, A. C., and Barrett, S. C. (2000). Evolution of floral display in *Eichhornia paniculata* (Pontederiaceae): direct and correlated responses to selection on flower size and number. *Evolution* 54, 1533–1545. doi: 10.1111/j.0014-3820.2000.tb00699.x

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Coloration of Flowers by Flavonoids and Consequences of pH Dependent Absorption

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Flavonoid pigments are key determinants of flower colors. As absorption spectra of flavonoids are known to be severely pH-dependent, cellular pH will play a crucial role in flower coloration. The flavonoids are concentrated in the vacuoles of the flowers' epidermal cells, and thus the pigments' absorption spectra are modulated by the vacuolar pH. Here we study the pH dependence of flavonoid absorption spectra in extracts from flowers of two poppy species *Papaver dubium* (red) and *Meconopsis cambrica* (orange), and a white and red *Mandevilla sanderi* variety. In the red poppy and *Mandevilla* flowers, absorption spectra of the cyanidin- and pelargonidin-based anthocyanins peak in the blue-green-wavelength range at low pH, but exhibit a distinct bathochromic shift at higher pH. This shift to longer wavelengths is not found for the blue-absorbing nudicaulin derivatives of *M. cambrica*, which have a similar absorption spectrum at low and high pH. The pH-dependent absorption changes of the white *M. sanderi*'s flavonoid remained restricted to the UV. An analysis of the spectra with logistic functions suggests that the pH-dependent characteristics of the basic states of flavonols and anthocyanins are related. The implications of tuning of pH and pigment absorption spectra for studies on flower color evolution are discussed.

Keywords: flavonol, anthocyanin, nudicaulin, carotenoid, pollination, *Papaver*, *Mandevilla*

INTRODUCTION

The plant kingdom harbors a remarkable diversity in flower colors. This colorful richness arose mostly because their coloration aid flowers in attracting pollinators, thereby enhancing the plants' reproductive success (van der Kooi and Ollerton, 2020). The visibility of a flower, and thus its attractiveness, is principally determined by the wavelength dependence of the fraction of incident light that is back-scattered. Whereas the backscattering is determined by the inhomogeneous structuring of the petals, the color of the backscattered light mainly depends on the flower's pigmentation (van der Kooi et al., 2016). Presumably, the pigment type is broadly correlated with pollination ecology, with flower colors being tuned to the visual system of the pollinators (Harborne and Smith, 1978; Chittka and Menzel, 1992; Brouillard and Dangles, 1994; de Camargo et al., 2019; Shrestha et al., 2019; van der Kooi et al., 2019).

The most common flower pigments are the carotenoids and flavonoids (Grotewold, 2006). The blue-absorbing carotenoids, e.g. zeaxanthin, carotene, and lutein, create yellow tissues, and the green-absorbing astaxanthin causes a red color (Yuan et al., 2002; Grotewold, 2006; Shafaa et al., 2007; Rasmussen et al., 2012). Of the flavonoids, the flavonols absorb virtually exclusively in the UV and thus cause white to pale-yellow colors. Widespread flower pigments are the spectrally variably absorbing anthocyanins, which can confer red, blue, or purple colors to plant tissues (e.g., Markham, 1982; Brouillard and Dangles, 1994). The anthocyanin pigments, glycosides of anthocyanidin aglycons, are secondary metabolites of land plants that can be biochemically detected in species as ancient as mosses (Bendz et al., 1962; Koes et al., 1994; Rausher, 2006; Campanella et al., 2014). The three major anthocyanin pigment types that are found in terrestrial plants are cyanidin-3-glycoside (brick red), pelargonidin-3-glycoside (orange/red), and delphinidin-3-glycoside (blue/purple), of which cyanidin is more present in primitive families, while delphinidin is restricted to the more highly evolved angiosperm plant families (Harborne and Williams, 2000).

Anthocyanin pigments are water soluble and concentrated in the epidermal cells, where the pigments are most effective in creating an intense coloration (van der Kooi et al., 2016). Importantly, the anthocyanins occur in the vacuoles, which often have a quite acidic pH (Pourcel et al., 2010; Passeri et al., 2016). The acidic vacuolar pH is probably intimately related to the severe pH dependence of the flavonoid absorption spectra (e.g., Jurasekova et al., 2014; Tang et al., 2019). The factors and mechanisms determining the vacuolar pH thus play a central role in flower coloration and are important for pollination.

We previously investigated how pigment absorption and scattering together determine the colors of the flowers of a few poppy species (van der Kooi and Stavenga, 2019; Dudek et al., 2020; Martínez-Harms et al., 2020). We thus found that the flowers of the common poppy (*Papaver rhoeas*) in the Middle East exhibit a low UV-reflectance due to a considerable amount of the flavonols kaempferol and quercetin, but those in Germany have much less of the flavonols and hence have a distinct UV reflectance (Dudek et al., 2020). The anthocyanins responsible for the bright red colors of *P. rhoeas* and the related long-headed poppy (*Papaver dubium*) are cyanidin and pelargonidin glycosides (Scott-Moncrieff, 1936; Acheson et al., 1956, 1962; Harborne, 1958; Dudek et al., 2020). The *in situ* absorbance spectra of the red flowers of the European *P. rhoeas* and *P. dubium* are very similar, but *P. dubium* appears to have a lower concentration of pelargonidin (Figure 6A in van der Kooi and Stavenga, 2019).

Previous investigations on the pH dependence of pigments mostly concentrated on single anthocyanins *in vitro*, or studied the influence of pH on the complex mechanisms of blue flower coloration (Mazza and Brouillard, 1987; Yoshida et al., 2009). Here we investigate how the absorbance spectra of the flavonoid pigments depend on pH. We chose two sets of species/varieties that are roughly similar in anatomy and backscattering, but differ in coloration due to different pigmentation. We thus compare flowers of *P. dubium* (red), the related Welsh poppy *Meconopsis cambrica* (orange), and two different-colored varieties of *Mandevilla sanderi* (also known as *Dipladenia*). We conclude that the vacuolar pH plays a crucial role for realizing strongly colored flowers with anthocyanins that are attractive for pollinators and discuss how pH is important for studies on the evolution of flower coloration.

MATERIALS AND METHODS

Plant Material and Photography

Long-headed poppies, *P. dubium*, were collected at road sides in Groningen, Netherlands. The *M. sanderi* plants, "Sundaville White" and "Sundaville Red," hereafter abbreviated as White and Red, were obtained from local suppliers. Welsh poppies, *M. cambrica*, were taken from a local garden. Macro-photographs of the flowers were made with a Canon EOS 7D.

Spectrophotometry of Flower Lobes and Extracts

Reflectance spectra of flower lobes were performed with a bifurcated reflection probe. The light source was a deuterium-halogen lamp [AvaLight-D(H)-S] and the spectrometer an AvaSpec-2048 (Avantes, Apeldoorn, Netherlands). Pigments were extracted from 1 to 5 cm^2-sized petal or lobe pieces in a ∼40 ml solution of 50:1 methanol: 1 M hydrochloric acid (MeOH and HCL purchased from Sigma Aldrich, Steinheim, Germany). Absorbance spectra of the extracted pigments were measured immediately after the extraction in 10 mm light path quartz cuvettes at room temperature. The pH of the extract, measured with a calibrated pH meter, was modified by adding adequate amounts of KOH solution. The pH-dependence of the absorbance (or optical density D), was evaluated at a few different wavelengths.

When a medium contains more than one pigment, and the concentration of one pigment changes due to the action of some agent (e.g., a change in pH), the different components of the process of the pigment changes can be separated by subtracting the constant background absorbance caused by the other pigments. When the absorbance increased with increasing pH the values were approximated by

$$D = \sum_{i}^{n} A_i F_i + B \tag{1a}$$

and when the absorbance decreased with increasing pH by

$$D = \sum_{i}^{n} A_i \left(1 - F_i\right) + B \tag{1b}$$

Here F_i is a logistic function, with parameter pK_i

$$F_i = 1/\left[1 + 10^{-(\mathrm{pH}-\mathrm{pK}_i)}\right] \tag{1c}$$

which accounts for n = 1 or 2 pH-dependent components with amplitude A_i and background B. We evaluated the absorbance changes at those critical wavelengths where the changes of

pigment states were large. The pK-values of the components resulted from fits of Eq. 1 to the measurements.

RESULTS

Papaver dubium

The flowers of the longheaded poppy, *P. dubium*, display an almost homogeneous, bright-red color (**Figure 1A**). However, absorbance spectra measured at the distal and proximal flower areas differ distinctly in amplitude as well as shape, which shows that the pigmentation causing the red color is not due to one pigment that is distributed inhomogeneously (**Figure 1B**).

We investigated the pigments in methanol extracts from different flowers of *P. dubium*, which yielded bright red solutions. **Figures 1C,D** show two exemplary cases where the measured absorbance spectra (exp) have rather different shapes, with peak wavelengths at 512 and 501 nm, respectively. These experimental spectra closely resemble the absorption spectra of cyanidin and pelargonidin glycosides, the two fundamental anthocyanidins that were isolated from flowers of the common poppy *P. rhoeas*, which have peak wavelengths 521 and 507 nm, respectively (**Figures 1C,D**; from Dudek et al., 2020). We hence conclude that *P. dubium* flowers contain variable amounts of cyanidin and pelargonidin derivatives, in agreement with previous studies (Scott-Moncrieff, 1936; Acheson et al., 1956, 1962).

We have added to **Figures 1C,D** the absorption spectra of quercetin and kaempferol glycosides, because they were also identified in *P. rhoeas* flowers (Dudek et al., 2020). These flavonols have the same hydroxylation pattern and are derived from the same precursor as the two anthocyanins. The contribution of the two flavonols to the measured absorbance spectra is clearly minor, in agreement with reflectance and transmittance spectra measured on intact *P. dubium* flowers, which also indicated that absorption in the UV is very moderate (see Figures 1E,F of van der Kooi and Stavenga, 2019).

The anthocyanin spectra severely depend on pH (**Figures 2**, **3**). **Figures 2A,B** show how the absorbance spectra of a flower extract (the case of the experimental spectrum of **Figure 1C**) change when the pH gradually increases. For pH < 5, the absorbance in the blue-green wavelength range decreases about proportionally (**Figure 2A**). The absorbance values assessed at the peak wavelength, 512 nm, are well approximated with a one-component logistic function (using Eqs 1b,c), yielding pK = 3.1 (**Figure 2C**), which suggests the pH-dependent transition of two pigment states.

For pH > 5, the absorbance in the ultraviolet (UV-A; peak wavelength ∼373 nm) as well as orange/red wavelength range (peak wavelength ∼631 nm) steadily increases. The absorbance values of the pH-dependent spectra at 373 nm approximated with a two-component logistic function (using Eqs 1a,c) yielded pK-values of 6.6 and 9.0. The absorbance values at 631 nm could also be well described by a two-component logistic function, yielding

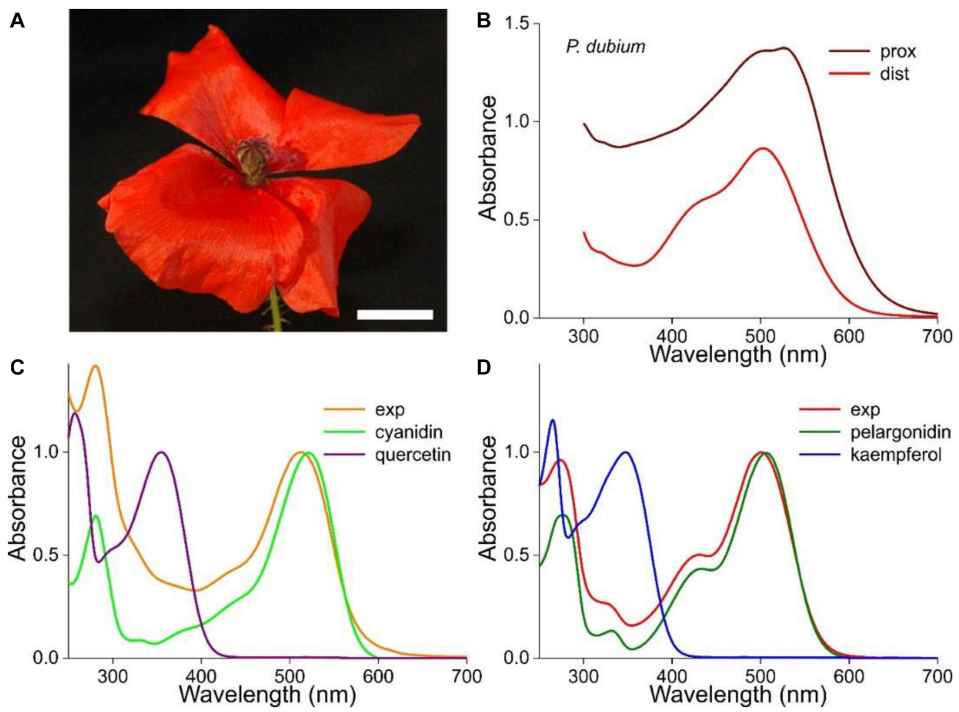


FIGURE 1 | Absorbance spectra of the long-headed poppy *Papaver dubium*. **(A)** A *P. dubium* flower; scale bar 1 cm. **(B)** Absorbance spectra of a proximal and distal petal area (from van der Kooi and Stavenga, 2019). **(C)** Absorbance spectrum of a flower extract (exp; normalized spectrum for pH = 2.5 of **Figure 2A**) compared with the spectrum of isolated cyanidin; the spectrum of quercetin is added. **(D)** Absorbance spectrum measured of another flower extract (exp; normalized spectrum for pH = 2.4 of **Figure 3A**) compared with the spectrum of isolated pelargonidin; the spectrum of kaempferol is added (flavonoid spectra from Dudek et al., 2020).

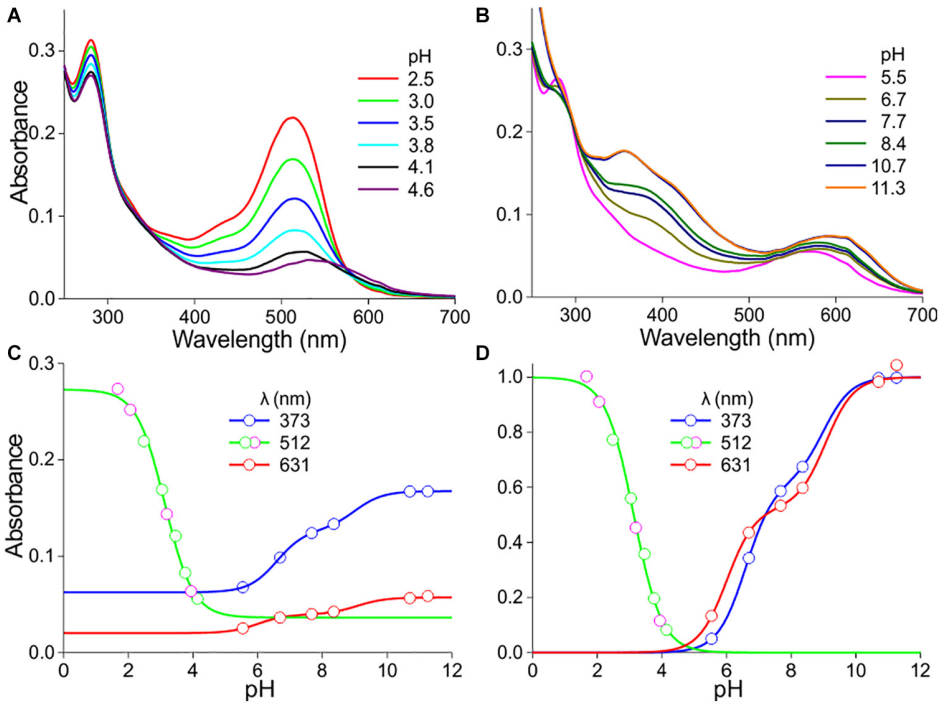


FIGURE 2 | pH dependence of *P. dubium* pigments. **(A)** Absorbance spectra of extract for pH < 5. **(B)** Absorbance spectra for pH > 5. **(C)** Absorbance values derived from **(A,B)** at a few wavelengths, fitted with logistic functions. **(D)** The data of **(C)** with background subtracted and normalized [green symbols in **(C,D)** derived from **(A)**, magenta symbols from another measurement series].

similar pK values: pK = 6.0 and 9.1 (**Figure 2C**). Subtraction of the background and subsequently normalizing the data of **Figure 2C** yielded **Figure 2D**.

Figures 3A,B show how the absorbance spectra of another flower extract (that of the experimental spectrum of **Figure 1D**) change when the pH gradually increases. For pH < 5, the absorbance values at the peak wavelength, 501 nm, fitted with a one-component logistic function, yielded pK = 3.2 (**Figure 3C**), very similar to the pK = 3.1 of **Figure 2C**. For pH > 5, fitting a two-component logistic function to the absorbance values at 355 nm yielded pK = 7.3 and 9.4, whilst those at 572 nm produced pK = 7.1 and 9.0 (**Figure 3C**). Subtraction of the background and subsequently normalizing the data of **Figure 3C** yielded **Figure 3D**.

The pK-values of the logistic functions in **Figures 2C,D**, **3C,D** appear to be very similar, which suggests that the same pH-dependent structural changes govern the absorbance changes of cyanidin and pelargonidin upon decreasing acidity or increasing alkalinity (Pina et al., 2012). To further investigate this, we performed the same experimental approach on extracts obtained from flowers of the White and Red Sundaville morphs of *M. sanderi* plants (**Figures 4A,B**).

Mandevilla sanderi

The flower lobes of *M. sanderi* "White Sundaville" are brightly white colored (**Figure 4A**), which might suggest the absence of pigments, but reflectance spectra show that this is certainly not the case (**Figure 4C**). The lobe's reflectance was indeed high throughout the visible wavelength range, but the low reflectance in the UV revealed the presence of UV-absorbing pigments; a common feature in (to humans) white flowers (Chittka et al., 1994; Kevan et al., 1996). Methanol extracts of the White flower's lobes had for pH < 5 virtually identical absorbance spectra, with peak wavelength ∼320 nm. At pH > 5, with increasing pH the absorbance increased and shifted bathochromically, peaking at 385 nm (**Figure 4D**). An isosbestic point at 337 nm suggested the existence of two pigment states with different absorption spectra. Absorbance difference spectra calculated by subtracting the absorbance spectrum of pH = 2.9 from the measured spectra indeed had a very similar shape (**Figure 4G**). **Figure 4J** shows normalized spectra of the low-pH (acid) and high-pH (alkaline) states. To further characterize this, we evaluated the absorbance spectra of **Figure 4D** at λ = 388 nm, the peak wavelength of the difference spectra (asterisks in **Figure 4M**). The data could only be well-fitted with a two-component logistic function (Eqs 1a,c), which yielded pK-values 6.9 and 8.8, thus indicating that more than two pigment states are involved.

The pigments extracted from the flower lobes of *M. sanderi* "Red Sundaville" (**Figure 4B**) behaved rather differently. At low pH-values, the absorbance spectrum featured two bands, peaking at 330 nm and 515 nm. Increasing the pH-value from 2.0 to 4.6 caused a severe drop of the main band in the green wavelength range (**Figure 4E**). To analyze this pH-dependent change, we calculated again absorbance difference spectra, by subtracting the absorbance spectrum measured at pH = 4.6 from the absorbance spectra measured at pH < 5. The resulting difference spectra were

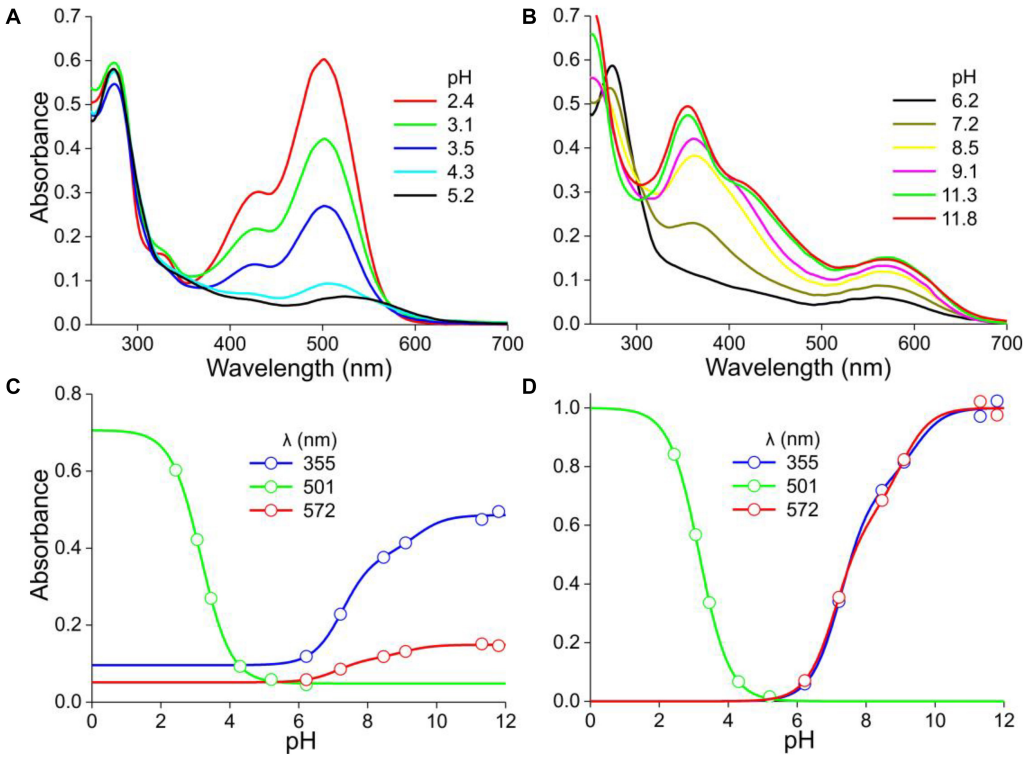


FIGURE 3 | Absorbance spectra of another *P. dubium* flower extract at various pH. **(A)** Absorbance spectra for pH < 6. **(B)** Absorbance spectra for pH > 6. **(C)** Absorbance values derived from **(A,B)** at a few wavelengths, fitted with logistic functions. **(D)** The data of **(C)** with background subtracted and normalized.

about proportional to each other, suggesting again a proportional change in a pigment state depending on the pH (**Figure 4H**). The average of the spectra of **Figure 4H**, which is shown normalized in **Figure 4K** (anth), closely resembles the cyanidin spectrum of **Figure 1C** (**Figure 4K**, cya).

We analyzed the pH-dependence of this pigment state by assessing the absorbance at its peak wavelength (515 nm) as a function of pH (**Figure 4N**). The pH-dependence could be well fitted with a single-component logistic function (Eqs 1b,c), yielding pK = 3.1, identical to the *P. dubium* case of **Figure 2**. This confirms that cyanidin is the prominent anthocyanin that determines the coloration of *M. sanderi* "Red Sundaville." Yet, **Figures 4E,H** clearly show that the flowers contain, in addition to the blue-green absorbing cyanidin, a UV-absorbing pigment. We, therefore, normalized the spectra of **Figure 4E**, subtracted the average anthocyanin spectrum (**Figure 4K**, anth) and then normalized the results. This yielded an absorbance spectrum peaking at 325 nm, which we tentatively assume to be due to flavonols (**Figure 4K**, flav). However, quite possibly the pigment may actually be another flavonoid, e.g., a flavone (Markham, 1982).

When the pH increased to above five, an absorbance band peaking around 600 nm gradually emerged. In addition, the UV-band shifted bathochromically, resulting in a prominent band peaking at 380 nm (**Figure 4F**), which resembles the absorbance band peaking at 380 nm that emerged with pH > 5 in the case of the White lobe's extract (**Figure 4D**). We furthermore analyzed the set of spectra again by subtracting the absorbance spectrum measured at pH = 5.8 from the other spectra measured at pH > 5, which yielded absorbance difference spectra with two absorbance bands with peak wavelengths 388 and 598 nm (**Figure 4I**).

We subsequently evaluated the absorbance spectra of **Figures 4E,F** at these peak wavelengths (**Figure 4N**). The absorbance values at 388 nm as a function of pH showed a biphasic behavior, with first a decline between pH = 2 and 5 (**Figure 4N**). This absorbance decline is apparently due to the fall in the cyanidin concentration (**Figure 4H**), as the data for pH < 5 were well approximated by Eqs 1b,c, using pK = 3.1. For pH > 5, the 388 nm data could be well fitted with Eqs 1a,c ($n = 2$), yielding pK = 6.4 and 8.4.

We finally analyzed the pH dependence of the long-wavelength absorbance band by estimating the absorbance at 598 nm as a function of pH and fitting Eqs 1a,c to the data, which yielded pK = 5.7 and 8.8 (**Figure 4N**). Subtracting the background and normalizing the data as before yielded **Figure 4O**. Taking the calculated pH-dependencies into account, we finally analyzed the difference spectra of **Figure 4I**, which produced the normalized absorbance spectrum of a UV- and a red-absorbing pigment (**Figure 4L**).

Meconopsis cambrica

The Welsh poppy, *M. cambrica*, features bright yellow or orange flowers. The normalized absorbance spectra of intact yellow and orange flowers have very similar shapes (**Figures 5A,B**),

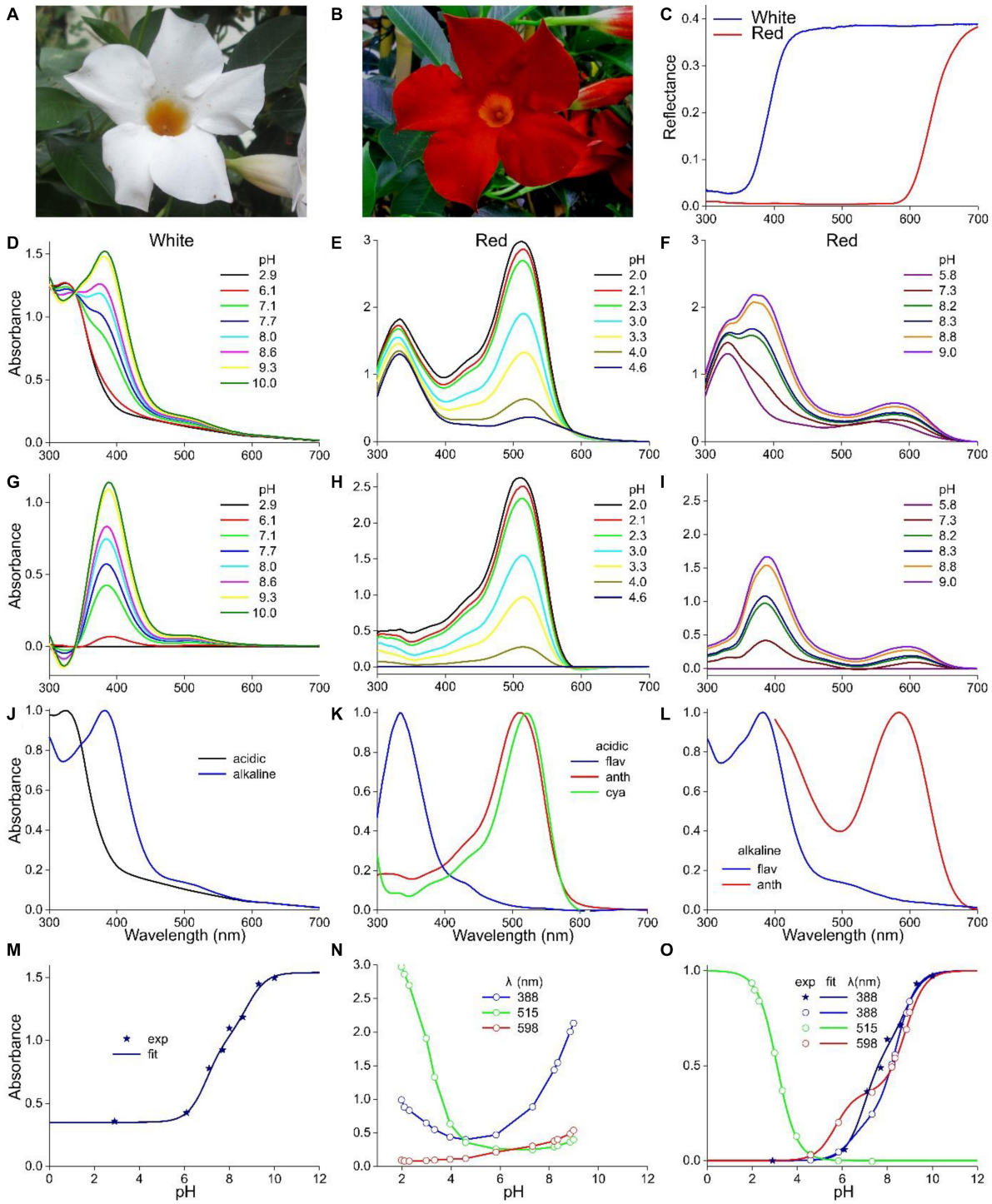


FIGURE 4 | *Mandevilla sanderi* flowers and absorbance spectra of extracted pigment at various pH values. **(A)** Flower of a White Sundaville morph with white lobe and yellow tube. **(B)** Flower of a Red Sundaville morph with red lobe and orange tube. **(C)** Reflectance spectra of the lobes of the flowers of panels **(A,B)**. **(D)** Absorbance spectra of pigment extracted from a White lobe. **(E)** Absorbance spectra of pigment extracted from a Red lobe, measured at low pH values. **(F)** as **(E)**, but spectra measured at higher pH-values. **(G)** Absorbance difference spectra obtained by subtracting the spectrum for pH = 2.9 from the spectra of panel **(D)**. **(H)** Absorbance difference spectra obtained by subtracting the spectrum for pH = 4.6 from the spectra of panel **(E)**. **(I)** Absorbance difference spectra obtained by subtracting the spectrum for pH = 5.8 from the spectra of panel **(F)**. **(J)** Normalized absorbance spectra of the White lobe in the acid and alkaline state. **(K)** Normalized absorbance spectra of flavonols and anthocyanins of the Red lobe in the acid state, together with the cyanidin spectrum of **Figure 1C**. **(L)** Normalized absorbance spectra of the flavonols and anthocyanins of the Red lobe in the alkaline state. **(M)** Absorbance values at 388 nm of the spectra of panel **(D)** as a function of pH (symbols), fitted with Eq. 1 (solid line). **(N)** Absorbance values at 388, 515, and 598 nm of the spectra of panels **(E,F)** as a function of pH. **(O)** Background subtracted and normalized absorbance values of panels **(M,N)** together with fits.

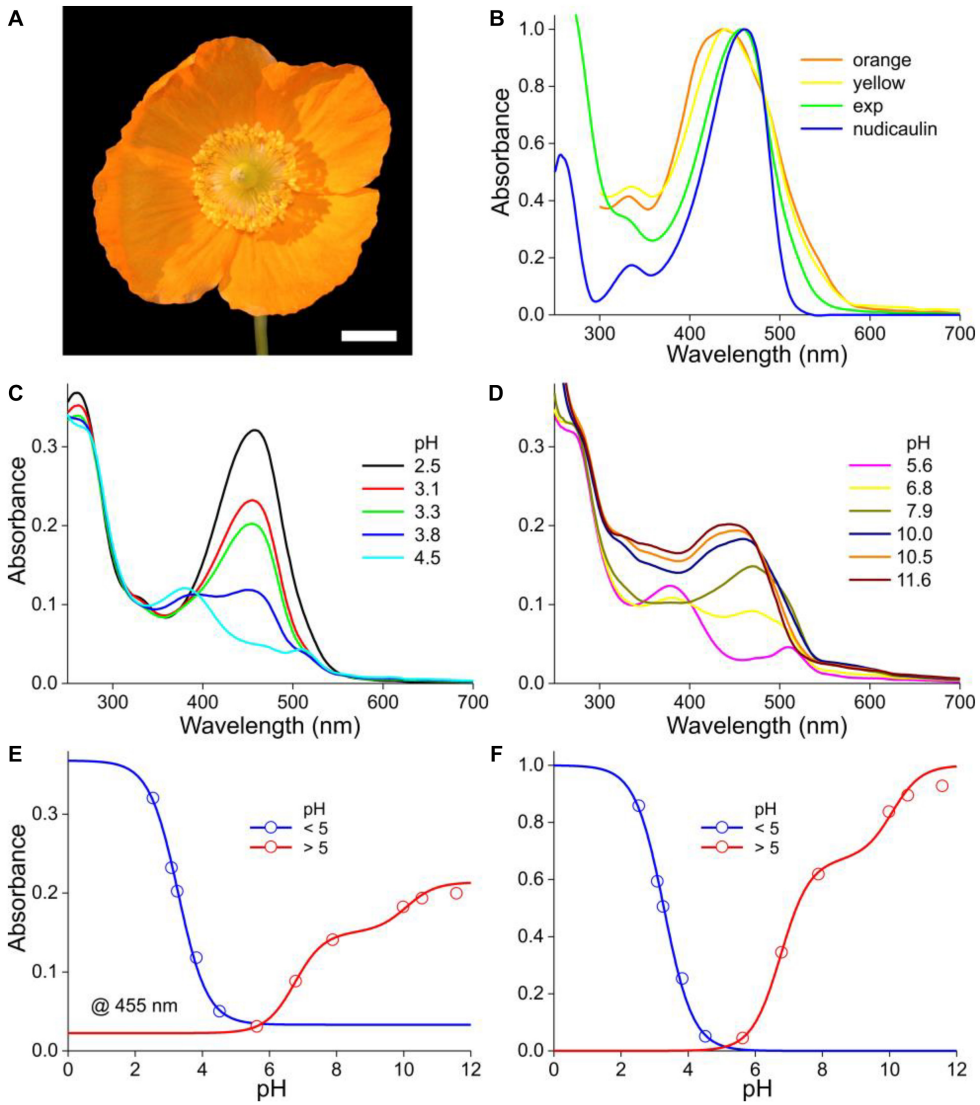


FIGURE 5 | The Welsh poppy *M. cambrica* and pH-dependent absorbance spectra. **(A)** A flower; scale bar 1 cm. **(B)** Normalized absorbance spectra of petals of orange and yellow morphs (from van der Kooi and Stavenga, 2019), the normalized spectrum of panel **(C)** for pH = 2.5 (exp), and that of nudicaulin (from Dudek et al., 2016). **(C)** Absorbance spectra for pH < 5. **(D)** Absorbance spectra for pH > 5. **(E)** Absorbance values derived from panels **(C,D)** at 455 nm (symbols), fitted with logistic curves. **(F)** The data of **(E)** with background subtracted and normalized.

suggesting the presence of the same pigment, but with the yellow flowers having a lower concentration (Figure 6B of van der Kooi and Stavenga, 2019). Yellow *M. cambrica* flowers are colored by nudicaulins (Tatsis et al., 2013), and therefore **Figure 5B** shows the absorption spectrum of nudicaulin glycoside isolated from *Papaver nudicaule* (from Dudek et al., 2016). It indeed closely resembles the absorbance spectrum of methanol extracts of orange flowers at low pH (**Figure 5B**, exp).

As in the previous cases, the absorbance of *M. cambrica* flower extracts decreases steadily with increasing pH (**Figure 5C**). Similar as in *P. nudicaule* petal extracts, between pH = 4 and 7 a minor absorbance band at 510 nm arises (Dudek et al., 2018). However, with increasing alkaline pH the absorbance band shifts again to lower wavelengths and rises in about the same wavelength range as that of the acid pigment state. The resulting absorbance spectra peak in the blue wavelength range at 460 nm at strongly acidic pH, at 510 nm at weakly acidic and at 445 nm at alkaline pH. To investigate the pH-dependence of the three phases, we assessed the absorbance values of the spectra of both **Figures 5C,D** at 455 nm. Fitting the pH < 5 data with a one-component logistic function yielded pK = 3.3, and fitting the pH > 5 data with a two-component logistic function yielded pK = 6.6 and 10.0. The absorbance spectra (or their difference spectra) could not be approximated with a restricted set of absorbance spectra similar to the cases treated above. A separate, more extensive approach will be necessary to unravel the multiple participating components. We also analyzed the yellow form of *M. cambrica*. It suffices to note here that the absorbance spectra

of extracts of yellow flowers showed a similar pH-dependence as that of the orange form, with only very slight spectral differences.

DISCUSSION

We have studied how floral pigment absorbance depends on pH in a few flowers with flavonoid and flavonoid-derived pigments. We chose two different poppy species that have a largely similar anatomy and backscattering but differ in pigmentation and coloration (van der Kooi and Stavenga, 2019). We furthermore investigated two *Mandevilla* varieties. Interpreting studies on cultivated plants from an ecological and evolutionary point of view needs caution, because it is hard to know what phenotypic traits have arisen in nature and have been selected by plant breeders. Nevertheless, cultivated plants can be useful to study specific optical properties, particularly when cultivated lines greatly vary in one specific trait. In the case of the studied *Mandevilla* plants, cultivation resulted in flowers of similar thickness and backscattering (two important aspects of flower coloration; van der Kooi et al., 2016), but with highly different types of pigmentation (**Figure 4C**). The two poppy species and the two *Mandevilla* varieties thus provide a valuable resource for studies on the fundamental pigmentation properties of flowers.

Absorbance spectra of methanol extracts generally corresponded well with absorbance spectra measured from intact petals, although slight differences in spectral shape were encountered in all studied cases. Detailed chemical analyses revealed that flowers contain a multitude of flavonoids. For instance, the flowers of the common corn poppy, *P. rhoeas*, contain several glycosides of the flavonols kaempferol and quercetin as well as of the anthocyanidins cyanidin and pelargonidin (Dudek et al., 2020). The diversity in the absorbance spectra thus is not surprising, as extracts differ in solvent and concentration from the vacuolar conditions and may thus alter associative effects between pigments and solvent. Glycosylation as well as acylation can distinctly modify the absorption spectrum of the flavonoids (Giusti et al., 1999; Dudek et al., 2016). Furthermore, substantial spectral shifts can be exerted by co-pigmentation (Mazza and Brouillard, 1990).

Considerable insight has been assembled concerning the biosynthesis, the genetics, and the evolution of flower pigments, especially of the carotenoids and anthocyanins (Koes et al., 1994; Mol et al., 1998; Grotewold, 2006; Rausher, 2006; Glover and Martin, 2012; Campanella et al., 2014), but detailed studies on the pH dependence of pigment absorbance spectra, especially of complex pigment compositions, are scarce. The absorbance spectra of the cyanidin and pelargonidin derivatives encountered in the extracts of *P. dubium* flowers severely depend on pH. For pH < 5, the pH dependence is well described by a declining, single-component logistic function with pK ~ 3. This conforms to the general characteristic of flavylium compounds, where the distinctly absorbing cation converts into colorless hemiketal and *cis*- and *trans*-chalcone forms (Mazza and Brouillard, 1990; Pina et al., 2012). At higher pH various anionic quinonoidal bases are created (Mazza and Brouillard, 1990; Pina et al., 2012; Rakić et al., 2015; Dangles and Fenger, 2018; Sigurdson et al., 2018), which process can be approximated with a two-component logistic function. The differences in the absorbance spectra of **Figures 2**, **3** in the long-wavelength range suggest that the absorption spectra of the quinonoidal bases depend on being cyanidin- or pelargonidin-based derivatives.

The absorbance spectra of the *M. sanderi* "Red Sundaville" flower extracts show in the visible wavelength range a similar pH-dependence as that of the cyanidin-dominated *P. dubium* (**Figures 3A,B**, **4E,F**), but the spectra in the UV-wavelength range differ considerably. We, therefore, investigated the flowers of the *M. sanderi* "White Sundaville," because they contain only UV-absorbing pigment (**Figures 4A,D**), having at low pH an absorbance peak wavelength of 325 nm and at high pH 382 nm (**Figure 4J**). We tentatively attribute this to flavonol, because its pH-dependence follows a two-component logistic function that is very similar to that of the anthocyanin. The same (or very similar) flavonoid pigment appears to exist in the Red Sundaville (**Figure 4O**).

Flavonoids have been identified as colorants in numerous flowers (Iwashina, 2003; Zhao and Tao, 2015). For instance, in methanol extracts of *Dipladenia martiana* flowers, the flavonols quercetin and kaempferol and several related components were identified (de Carvalho et al., 2001). The spectral analysis of the investigated *Mandevilla* (*Dipladenia*) flowers suggests that the different types of pigment (flavonols in the White morph versus flavonols and anthocyanins in the Red morph) behave similarly with regard to pH changes.

The Welsh poppy, *M. cambrica*, is a special case as its flowers are colored by nudicaulins, an unusual group of indole alkaloids, which are generated by combining anthocyanins with an indole (Tatsis et al., 2013; Dudek et al., 2016, 2018; Devlin and Sperry, 2020). We note that the pH-dependent processes of *M. cambrica*'s nudicaulin can be described by logistic functions with very similar pK-values as those of the anthocyanins (**Table 1**). The nudicaulin pigments were analyzed in great detail in the Iceland poppy, *P. nudicaule* (Tatsis et al., 2013; Dudek et al., 2016, 2018). Interestingly, the yellow stamens of the yellow flowers contained carotenoids and not nudicaulins (Dudek et al., 2016). Yellow flowers generally contain carotenoids, which is also the case with the *M. sanderi* flowers, where carotenoids are expressed in the flower tube (see **Figures 4A,B**). In the yellow/orange-colored

TABLE 1 | Summary of the pK-values obtained for the pH-dependent processes in the studied flower extracts.

Flower	pH < 5	pH > 5 (λ < 500 nm)	pH > 5 (λ > 500 nm)
P. dubium 1 (**Figure 2**)	3.1	6.6/9.0	6.0/9.1
P. dubium 2 (**Figure 3**)	3.2	7.3/9.4	7.1/9.0
White *M. sanderi* (**Figure 4**)	–	6.9/8.8	–
Red *M. sanderi* (**Figure 4**)	3.1	6.4/8.4	5.7/8.8
M. cambrica (**Figure 5**)	3.3	6.6/10.0	–

For pH < 5, a one-component logistic function was fitted to the absorbance changes, while for pH > 5 in both the short- and long-wavelength ranges a two-component logistic function was used.

flowers of *M. cambrica* we did not obtain convincing evidence for anthocyanins.

The absorption spectra of floral pigments strongly depend on pH, which has severe consequences for flower coloration. The important question then is to know the vacuolar conditions in the flowers. Measurements of the pH of flower epidermal cells yielded values varying between 2.5 in a begonia cultivar and 7.5 in morning glory cv. Heavenly Blue (Stewart et al., 1975). Curiously, in the epidermal cells of flowers whose only anthocyanins were cyanidin glycosides, the associated colors widely varied, between strong pink, deep red, purple, and even moderate blue, whereas the pH ranged from 3.1 to 5.5 (Stewart et al., 1975). In this pH range, the anthocyanins' absorption spectra have similar shapes. The absorption amplitude is at most half of the maximal value, and at higher pH-values it further diminishes. A solution to the loss in absorption upon increasing pH is co-pigmentation, which stabilizes the acidic pigment state and can modify the spectral absorption (Mazza and Brouillard, 1990).

The anthocyanins are concentrated in the epidermal cells' vacuoles, and therefore a crucial factor determining the flower's color is the vacuole's pH, which is highly dependent on an H^+-ATPase (Verweij et al., 2008; Yoshida et al., 2009). Minor changes in pH can cause major changes in coloration. Indeed, a pH change from 3.3 to 4.0 in the vacuoles in *Hydrangea macrophylla* sepals makes the color shift from blue to red (Yoshida et al., 2003). A similar color change occurs in the morning glory cv. Heavenly Blue during the flower-opening period, but it is here due to an unusual increase in vacuolar pH from 6.6 to 7.7 acting on a tricaffeoylated anthocyanin, the heavenly blue anthocyanin (Yoshida et al., 2009). We conclude that for the flowers studied in the present paper, and probably for anthocyanin- and flavonol-based colors more broadly, vacuolar pH crucially determines the pigment absorbance spectrum.

An intriguing question is how vacuolar pH, pigment absorbance spectra, and structural aspects of flowers are tuned to optimize visibility to (local) pollinators. Previous studies demonstrated that regional differences exist in the coloration of *P. rhoeas*, which can be linked to the color vision of local pollinators, beetles in the Middle East, and bees in Europe (Dudek et al., 2020; Martínez-Harms et al., 2020). Also, *Mandevilla* flowers are pollinated by different groups of pollinators, including bees, butterflies, and moths (De Araújo et al., 2014; Rubini Pisano et al., 2019), which have different visual systems (van der Kooi et al., 2021). When considering the ultimate question of how flower colors are tuned to the visual system of their pollinators, disentangling the different structural as well as pigmentary aspects that create the flower's coloration is important.

We previously showed that in addition to pigments, the reflection and scattering properties of the flower structures also modify the coloration, and that scattering and pigmentation properties may be tuned for visual signaling to pollinators (van der Kooi et al., 2016, 2019; Stavenga et al., 2020). The emerging picture is that flowers have a wide gamut of possibilities to tune their coloration. A simple possibility is demonstrated by the yellow and orange flowers of *M. cambrica* that merely differ in concentration of essentially the same pigment (Figure 6B of van der Kooi and Stavenga, 2019). That subtle pH changes can greatly change the absorption spectrum of common flower pigments is important for studies on flower color evolution. The flower's absorbance can be further modified by other factors as co-pigmentation, self-association, and metal complexation. As flowers are the advertisement flags for pollinators, the spectral properties of the floral pigments are presumably tuned to optimize visibility. In comparison, the photoreceptor spectral sensitivities of the color vision systems of bees and birds are highly constrained to virtually fixed spectral values (Hart and Vorobyev, 2005; van der Kooi et al., 2021). Plants thus have evolved a much greater flexibility in tuning the display of their flowers than the flexibility their pollinators have in adjusting their color discrimination system.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS performed the analysis, made figures, and wrote the manuscript. HL executed the chemical analysis. BD and CK gave crucial input to the final manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Acheson, R., Harper, J., and McNaughton, I. (1956). Distribution of anthocyanin pigments in poppies. *Nature* 178, 1283–1284. doi: 10.1038/1781283a0

Acheson, R., Jenkins, C., Harper, J. L., and McNaughton, I. (1962). Floral pigments in *Papaver* and their significance in the systematics of the genus. *New Phytol.* 61, 256–260. doi: 10.1111/j.1469-8137.1962.tb06294.x

Bendz, G., Martensson, O., and Terenius, L. (1962). Moss pigments. *Acta Chem. Scand.* 16, 1183–1190. doi: 10.3891/acta.chem.scand.16-1183

Brouillard, R., and Dangles, O. (1994). "Flavonoids and flower colour," in *The Flavonoids*, ed. J. B. Harborne (London: Chapman and Hall), 565–588. doi: 10.1007/978-1-4899-2911-2_13

Campanella, J. J., Smalley, J. V., and Dempsey, M. E. (2014). A phylogenetic examination of the primary anthocyanin production pathway of the plantae. *Bot. Stud.* 55:10.

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181.

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Dangles, O., and Fenger, J. (2018). The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules* 23:1970. doi: 10.3390/molecules23081970

De Araújo, L., Quirino, Z., and Machado, I. (2014). High specialisation in the pollination system of *Mandevilla tenuifolia* (J.C. Mikan) Woodson (Apocynaceae) drives the effectiveness of butterflies as pollinators. *Plant Biol.* 16, 947–955. doi: 10.1111/plb.12152

de Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

de Carvalho, M. G., Carvalho Cranchi, D., Kingston, D. G., and Werle, A. A. (2001). Proposed active constituents of *Dipladenia martiana*. *Phytother. Res.* 15, 715–717. doi: 10.1002/ptr.865

Devlin, R., and Sperry, J. (2020). The curious yellow colouring matter of the Iceland poppy. *Org. Biomol. Chem.* 18, 5278–5286. doi: 10.1039/d0ob01162b

Dudek, B., Schneider, B., Hilger, H. H., Stavenga, D. G., and Martínez-Harms, J. (2020). Highly different flavonol content explains geographic variations in the UV reflecting properties of flowers of the corn poppy, *Papaver rhoeas* (Papaveraceae). *Phytochemistry* 178:112457. doi: 10.1016/j.phytochem.2020.112457

Dudek, B., Schnurrer, F., Dahse, H., Paetz, C., Warskulat, A., Weigel, C., et al. (2018). Formation of nudicaulins in vivo and in vitro and the biomimetic synthesis and bioactivity of O-methylated nudicaulin derivatives. *Molecules* 23:3357. doi: 10.3390/molecules23123357

Dudek, B., Warskulat, A., and Schneider, B. (2016). The occurrence of flavonoids and related compounds in flower sections of *Papaver nudicaule*. *Plants* 5:28. doi: 10.3390/plants5020028

Giusti, M. M., Rodríguez-Saona, L. E., and Wrolstad, R. E. (1999). Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* 47, 4631–4637. doi: 10.1021/jf981271k

Glover, B. J., and Martin, C. (2012). Anthocyanins. *Curr. Biol.* 22, R147–R150.

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Harborne, J. B. (1958). Spectral methods of characterizing anthocyanins. *Biochem. J.* 70, 22–28. doi: 10.1042/bj0700022

Harborne, J. B., and Smith, D. M. (1978). Correlations between anthocyanin chemistry and pollination ecology in the Polemoniaceae. *Biochem. Syst. Ecol.* 6, 127–130. doi: 10.1016/0305-1978(78)90038-8

Harborne, J. B., and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry* 55, 481–504. doi: 10.1016/s0031-9422(00)00235-1

Hart, N. S., and Vorobyev, M. (2005). Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *J. Comp. Physiol. A* 191, 381–392. doi: 10.1007/s00359-004-0595-3

Iwashina, T. (2003). Flavonoid function and activity to plants and other organisms. *Biol. Sci. Space* 17, 24–44. doi: 10.2187/bss.17.24

Jurasekova, Z. C., Domingo, C., Garcia-Ramos, J. V., and Sanchez-Cortes, S. (2014). Effect of pH on the chemical modification of quercetin and structurally related flavonoids characterized by optical (UV-visible and Raman) spectroscopy. *Phys. Chem. Chem. Phys.* 16:12802. doi: 10.1039/c4cp00864b

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1:252. doi: 10.1016/1360-1385(96)20008-1

Koes, R. E., Quattrocchio, F., and Mol, J. N. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *Bioessays* 16, 123–132. doi: 10.1002/bies.950160209

Markham, K. R. (1982). *Techniques of Flavonoid Identification*. London: Academic Press.

Martínez-Harms, J., Hadar, R., Márquez, N., Menzel, R., Shmida, A., Stavenga, D. G., et al. (2020). Enhanced UV-reflection facilitated a shift in the pollination system of the red poppy, *Papaver rhoeas (*Papaveraceae). *Plants* 9:927. doi: 10.3390/plants9080927

Mazza, G., and Brouillard, R. (1987). Color stability and structural transformations of cyanidin 3,5-diglucoside and four 3-deoxyanthocyanins in aqueous solutions. *J. Agric. Food Chem.* 35, 422–426. doi: 10.1021/jf00075a034

Mazza, G., and Brouillard, R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry* 29, 1097–1102. doi: 10.1016/0031-9422(90)85411-8

Mol, J., Grotewold, E., and Koes, R. (1998). How genes paint flowers and seeds. *Trends Plant Sci.* 3, 212–217. doi: 10.1016/s1360-1385(98)01242-4

Passeri, V., Koes, R., and Quattrocchio, F. M. (2016). New challenges for the design of high value plant products: stabilization of anthocyanins in plant vacuoles. *Front. Plant Sci.* 7:153. doi: 10.3389/fpls.2016.00153

Pina, F., Melo, M. J., Laia, C. A., Parola, A. J., and Lima, J. C. (2012). Chemistry and applications of flavylium compounds: a handful of colours. *Chem. Soc. Rev.* 41, 869–908. doi: 10.1039/c1cs15126f

Pourcel, L., Irani, N. G., Lu, Y., Riedl, K., Schwartz, S., and Grotewold, E. (2010). The formation of anthocyanic vacuolar inclusions in *Arabidopsis thaliana* and implications for the sequestration of anthocyanin pigments. *Mol. Plant* 3, 78–90. doi: 10.1093/mp/ssp071

Rakić, V. P., Ota, A. M., Skrt, M. A., Miljković, M. N., Kostić, D. A., Sokolović, D. T., et al. (2015). Investigation of fluorescence properties of cyanidin and cyanidin 3-O-β-glucopyranoside. *Hem. Ind.* 69, 155–163. doi: 10.2298/hemind140203030r

Rasmussen, H. M., Muzhingi, T., Eggert, E. M., and Johnson, E. J. (2012). Lutein, zeaxanthin, meso-zeaxanthin content in egg yolk and their absence in fish and seafood. *J. Food Comp. Anal.* 27, 139–144. doi: 10.1016/j.jfca.2012.04.009

Rausher, M. D. (2006). "The evolution of flavonoids and their genes," in *The Science of Flavonoids*, ed. E. Grotewold (New York, NY: Springer), 175–211. doi: 10.1007/0-387-28822-8_7

Rubini Pisano, A., Moré, M., Cisternas, M. A., Raguso, R. A., and Benitez-Vieyra, S. (2019). Breakdown of species boundaries in *Mandevilla*: floral morphological intermediacy, novel fragrances and asymmetric pollen flow. *Plant Biol.* 21, 206–215. doi: 10.1111/plb.12924

Scott-Moncrieff, R. (1936). A biochemical survey of some Mendelian factors for flower colour. *J. Genet.* 32, 117–170. doi: 10.1007/bf02982506

Shafaa, M. W. I., Diehl, H. A., and Socaciu, C. (2007). The solubilisation pattern of lutein, zeaxanthin, canthaxanthin and β-carotene differ characteristically in liposomes, liver microsomes and retinal epithelial cells. *Biophys. Chem.* 129, 111–119. doi: 10.1016/j.bpc.2007.05.007

Shrestha, M., Burd, M., Garcia, J. E., Dorin, A., and Dyer, A. G. (2019). Colour evolution within orchids depends on whether the pollinator is a bee or a fly. *Plant Biol.* 21, 745–752. doi: 10.1111/plb.12968

Sigurdson, G. T., Tang, P., and Giusti, M. M. (2018). Cis–trans configuration of coumaric acid acylation affects the spectral and colorimetric properties of anthocyanins. *Molecules* 23:598. doi: 10.3390/molecules23030598

Stavenga, D. G., Staal, M., and van der Kooi, C. J. (2020). Conical epidermal cells cause reduced velvety colouration and enhanced patterning in *Mandevilla* flowers. *Faraday Discuss.* 223, 98–106. doi: 10.1039/d0fd00055h

Stewart, R. N., Norris, K. H., and Asen, S. (1975). Microspectrophotometric measurement of pH and pH effect on color of petal epidermal cells. *Phytochemistry* 14, 937–942. doi: 10.1016/0031-9422(75)85162-4

Tang, B., He, Y., Liu, J., Zhang, J., Li, J., Zhou, J., et al. (2019). Kinetic investigation into pH-dependent color of anthocyanin and its sensing performance. *Dyes Pigm.* 170:107643. doi: 10.1016/j.dyepig.2019.107643

Tatsis, E. C., Böhm, H., and Schneider, B. (2013). Occurrence of nudicaulin structural variants in flowers of papaveraceous species. *Phytochemistry* 92, 105–112. doi: 10.1016/j.phytochem.2013.04.011

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B* 283:20160429. doi: 10.1098/rspb.2016.0429

van der Kooi, C. J., and Ollerton, J. (2020). The origins of flowering plants and pollinators. *Science* 368, 1306–1308. doi: 10.1126/science.aay3662

van der Kooi, C. J., and Stavenga, D. G. (2019). Vividly coloured poppy flowers due to dense pigmentation and strong scattering in thin petals. *J. Comp. Physiol. A* 205, 363–372. doi: 10.1007/s00359-018-01313-1

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belusic, G., and Kelber, A. (2021). Evolution of insect colour vision – from spectral sensitivity to visual ecology. *Ann. Rev. Entomol.* in press

Verweij, W., Spelt, C., Di Sansebastiano, G., Vermeer, J., Reale, L., Ferranti, F., et al. (2008). An H^+ P-ATPase on the tonoplast determines vacuolar pH and flower colour. *Nat. Cell Biol.* 10, 1456–1462. doi: 10.1038/ncb1805

Yoshida, K., Mori, M., and Kondo, T. (2009). Blue flower color development by anthocyanins: from chemical structure to cell physiology. *Nat. Prod. Rep.* 26, 884–915. doi: 10.1039/b800165k

Yoshida, K., Toyama-Kato, Y., Kameda, K., and Kondo, T. (2003). Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant Cell Physiol.* 44, 262–268. doi: 10.1093/pcp/pcg033

Yuan, J., Chen, F., Liu, X., and Li, X. (2002). Carotenoid composition in the green microalga *Chlorococcum*. *Food Chem.* 76, 319–325. doi: 10.1016/s0308-8146(01)00279-5

Zhao, D., and Tao, J. (2015). Recent advances on the development and regulation of flower color in ornamental plants. *Front. Plant Sci.* 6:261. doi: 10.3389/fpls.2015.00261

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Floral Color Properties of Serpentine Seep Assemblages Depend on Community Size and Species Richness

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Functional traits, particularly those that impact fitness, can shape the ecological and evolutionary relationships among coexisting species of the same trophic level. Thus, examining these traits and properties of their distributions (underdispersion, overdispersion) within communities can provide insights into key ecological interactions (e.g., competition, facilitation) involved in community assembly. For instance, the distribution of floral colors in a community may reflect pollinator-mediated interactions between sympatric plant species, and the phylogenetic distribution of color can inform how evolutionary contingencies can continue to shape extant community assemblages. Additionally, the abundance and species richness of the local habitat may influence the type or strength of ecological interactions among co-occurring species. To evaluate the impact of community size and species richness on mechanisms shaping the distribution of ecologically relevant traits, we examined how floral color (defined by pollinator color vision models) is distributed within co-flowering assemblages. We modeled floral reflectance spectra of 55 co-flowering species using honeybee (*Apis mellifera*) and syrphid fly (*Eristalis tenax*) visual systems to assess the distributions of flower color across 14 serpentine seep communities in California. We found that phylogenetic relatedness had little impact on the observed color assemblages. However, smaller seep communities with lower species richness were more overdispersed for flower color than larger, more species-rich communities. Results support that competitive exclusion could be a dominant process shaping the species richness of flower color in smaller-sized communities with lower species richness, but this is less detectable or overwhelmed by other processes at larger, more speciose communities.

Keywords: community assembly, pollinator color vision, pollinator-mediated competition, floral color, cognitive pollination ecology

INTRODUCTION

Competition for local resources like soil nitrogen and larger-scale factors such as climate have historically been documented as driving forces of plant community assembly (Webb, 2000; Fargione et al., 2003), but the persistence of a plant species in a community is contingent upon effective fertilization and seed production, which is mediated by animal pollinators for most angiosperms (Ollerton et al., 2011). Pollinators select flowers based on a variety of visual and olfactory cues and therefore have the potential to shape floral signal diversity in plant communities (Waser, 1986; Gumbert et al., 1999). Incorporating cognitive pollination ecology into plant community assembly studies is thus likely to prove fruitful for understanding the importance of plant–pollinator interactions and pollinator-mediated selection in flowering plant communities (Sargent and Ackerly, 2008; Schiestl and Johnson, 2013; Leonard and Masek, 2014; E-Vojtkó et al., 2020). In particular, there is a growing body of literature that has incorporated insights from pollinator vision to better understand the distribution of floral color in communities (de Jager et al., 2011; Dyer et al., 2012; Binkenstein et al., 2013; Shrestha et al., 2013; Burd et al., 2014; Muchhala et al., 2014; Makino and Yokoyama, 2015; Kemp et al., 2019; Shrestha et al., 2019).

Plant–pollinator interactions can shape the distribution of floral traits through their involvement in processes like competition or facilitation for visitation (Webb et al., 2002; Sargent and Ackerly, 2008). Such mechanisms may counteract or exacerbate abiotic processes such as habitat filtering (Ackerly, 2003) or stochastic processes such as neutral assembly (Hubbell, 2001). Ecological competition and facilitation are considered to operate at local spatial scales (Cavender-Bares et al., 2006). Examples of competitive exclusion shaping floral color assembly involve co-flowering plants competing for pollinators. In particular, selection may favor distinctiveness in floral coloration relative to other community members in a co-flowering assemblage, as this may aid in recognition by pollinators and support pollinator fidelity (Chittka, 1997; Gumbert et al., 1999; McEwen and Vamosi, 2010; Muchhala et al., 2014). This outcome would produce trait overdispersion of floral color (Sargent and Ackerly, 2008). Alternatively, facilitation may occur where one or more co-flowering species enhance another species' reproductive success. A mechanism of facilitation involves one (or both) co-flowering species enhancing pollinator visitation to the other due to their high similarity in floral color, which can enhance perceived floral abundance or other attraction for pollinators more so than a single species could produce alone (Rathcke, 1983; Bruno et al., 2003; Moeller, 2004; Ghazoul, 2006). Habitat filtering or ecological facilitation may produce a pattern of trait underdispersion (clustering) at the local habitat scale, dependent upon phylogenetic constraint (Sargent and Ackerly, 2008). Alternatively, high similarity in floral color between species may be a product of Batesian mimicry, where an unrewarding co-flowering species offers sensory cues (e.g., floral color) highly similar to its co-flowering model, or Müllerian mimicry, where both flowering species offer rewards and collectively offer a greater advertising display of flower (Benitez-Vieyra et al., 2007). In addition, evaluating phylogenetic community structure is crucial for inferring ecological mechanisms producing trait community structure (Webb et al., 2002; Wolowski et al., 2017), as phylogenetic inertia can serve as a source of constraint on community assemblage (van der Niet and Johnson, 2012).

In studies to date, both overdispersion and underdispersion of floral color have been documented in flowering communities (e.g., overdispersion: Muchhala et al., 2014; Makino and Yokoyama, 2015; underdispersion: McEwen and Vamosi, 2010; Kemp et al., 2019). At local spatial scales where competition and facilitation are considered to occur, habitat availability (e.g., the amount of inhabitable area) and species richness of local habitat may influence the type and strength of ecological interactions observed in plant–pollinator community assemblage, but these factors have been understudied (Rathcke, 1983; E-Vojtkó et al., 2020). Understanding the impact that habitat availability and species richness has on pattern interpretation could provide better context for inference of these ecological mechanism(s) (Cavender-Bares et al., 2006; Kraft et al., 2007).

To investigate signatures of overdispersion or clustering in the distribution of flower color across communities with varying habitat availability and species richness, we studied the assemblages of co-flowering plant species in the serpentine seeps of northern California. Seeps are an excellent model for studying questions of community assembly due to their metacommunity structure and constricted window of flowering time (Harrison et al., 2000; Freestone and Harrison, 2006). With this metacommunity, we asked: (A) Are co-flowering assemblages more or less diverse in floral color as viewed by common flower visitors compared to random expectation? (B) Is there an effect of seep area, plant density, and plant species richness on the floral color trait dispersion as viewed by common flower visitors? (C) Is there phylogenetic structure in the co-flowering communities?

MATERIALS AND METHODS

Study System

We studied the floral assemblages of 14 serpentine seeps and the greater regional pool of co-flowering plant species at the McLaughlin Natural Reserve in northern California, United States (38°51′029.45″N, 122°24′033.49″W). In particular, seeps are tributaries of creeks and are characterized by the water that flows slightly below the ground surface, creating a wet soil environment for much of the dry season in northern California (Harrison et al., 2000). The unique plant communities of each seep occur within a matrix of chaparral and grassland, and seeps are composed of bare, rocky outcrops interspersed with more suitable microclimates for plant colonization and growth (Kruckeberg, 1984). Seeps in our study varied in area, from 0.04 to 0.55 km^2, with an average area of 0.24 km^2. Seeps were located at a minimum of 0.08 km and up to 13.57 km apart, with a mean distance of 4.24 km apart from one another (**Figure 1**). Surveys of co-flowering plant assemblages were conducted at each of the 14 seeps during June and July 2013, and these served as the "observed" local co-flowering species assemblages

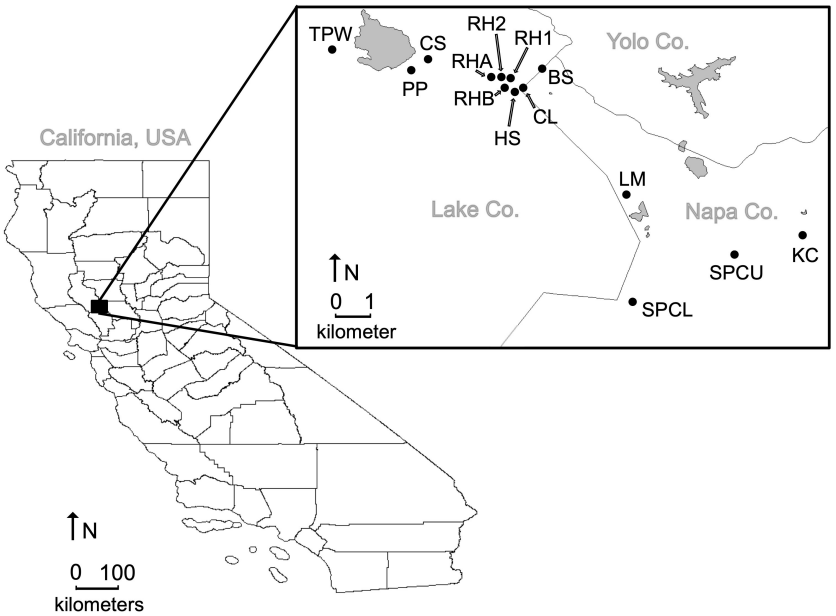


FIGURE 1 | Spatial distribution of serpentine seep communities (black circles) at McLaughlin Natural Reserve in Lake and Napa Counties, California, United States. Gray polygons represent bodies of water.

in our study. We also gathered floral spectra and insect visitation information about the broader "regional pool" of all co-flowering plant species across the entire serpentine seep metacommunity for use in constructing null models to compare with the observed communities (described in sections "Trait Community Structure Analyses" and "Phylogenetic Community Structure Analyses"). The inclusion of plant species in the broader regional pool was based on surveys performed in various seeps and immediately adjacent grassland in 2010, 2011, and 2013 (Alonso et al., 2013; Koski et al., 2015; **Supplementary Table S1**).

Site Surveys

To observe local species assemblages to compare with the regional species pool, site surveys were performed once for each site in the peak seep flowering period spanning June and July of 2013. To estimate values of habitat availability, we estimated seep area as well as plant density in the seeps. Plant density at each site was evaluated to account for how much of the seep was habitable for plant colonization and growth compared to the amount of uninhabitable, rocky outcrops that also compose serpentine seeps (Kruckeberg, 1984; Wolf et al., 1999). To estimate seep area, seep length and average seep width were measured. A transect line was laid along the longest axis of each seep. The length of the seep was measured along this line, and the width of the seep was measured at five different points at roughly even increments along the length of the seep to find the average width of seep. Along the transect line of the seep length, a transect tape was placed in the center of the seep running parallel to the transect line. To estimate values of plant species richness and plant density, at every 0.25 m of the transect tape, we documented whether the tape was over bare ground (soil or rock) or in contact with a plant. If it was in contact with a plant, the plant was identified to species. Species richness was calculated as the number of different species occurring in the survey of each seep. Additionally, to survey plants growing at the edge of the seep that may prefer slightly different microhabitat than in the center of the seep outcrops, a transect line was run in parallel to the edge of the seep length either on the east or south side of the seep (depending on how the length of the seep was oriented in the field). The seep edge was surveyed in the same manner as the seep length transect line. Each site was surveyed in this manner for up to 100 m. If a seep was longer than 100 m, then 50 m of the survey was conducted at the ends of the longest axis of the seep (25 m at each end) and 50 m of the survey were conducted in the middle of the seep.

Quantification of Seep Habitat: Area, Plant Density, and Species Richness

Seep area, plant density, and plant species richness within each seep were significantly and highly positively correlated (**Supplementary Table S2**), and we therefore consolidated these variables using principal component analysis (PCA). The first principal component, PC1, explained 75% of the variation in data. Negative loadings of PC1 indicated a small area, low plant density, and low species richness, whereas positive loadings of PC1 referred to larger, denser, and species-rich seep communities (**Supplementary Table S3** and **Supplementary Figure S1**). We utilized PC1 as a summary that we define as "seep index."

Collection of Floral Spectra, Background Spectra, and Irradiance

Of 63 regional co-flowering species known to be present in seeps or directly adjacent to seeps, we collected reflectance spectra

from 55 species (**Supplementary Table S1** and **Supplementary Figure S2**). These reflectance spectra were collected during the 2012 and 2013 field seasons in the months of June and July. We measured floral reflectance spectra from five different individual plants for 45 species, four different individual plants for one species (*Plagiobothrys stipitatus*), three different individual plants for three species (*Acmispon parviflorus*, *Allium amplectans*, and *Antirrhinum cornutum)*, two measurements from different individual plants for two species (*Hesperolinon disjunctum* and *Mimulus layneae*), and one individual plant for four species (*Heterocodon rariflorum*, *Lagophylla minor*, *Sisyrinchium bellum*, and *Collinsia sparsiflora*). Of the 63 species in the regional pool, the following eight remaining species could not be measured for color: *Allium falcifolium*, *Calochortus luteus*, *Clarkia purpurea*, *Euphorbia* sp., *Lactuca saligna*, *Linanthus* sp., *Plantago erecta*, and *Triteleia laxa*. All eight species had been documented sporadically in seep communities and adjacent grassland in either 2010 or 2011 (unpublished data), and they were not recorded during seep surveys in 2013. We therefore exclude these eight species for community trait analysis and community phylogenetic analysis.

Across all reflectance spectra collected in 2012 and 2013, three spectrometers were utilized (USB2000+, USB4000, and Jaz, Ocean Optics, Dunedin, FL, United States; species-specific details in **Supplementary Table S4**). Spectra were collected using either an internal pulsed-xenon light source (Jaz, Ocean Optics, Dunedin, FL, United States) or a deuterium–halogen light source (DH-2000-BAL, Ocean Optics, Dunedin, FL, United States) with a Spectralon white standard (Labsphere, North Sutton, NH) and dark correction to measure percent reflectance from 300 to 700 nm, which is the general range of color perception by many flower-visiting insects, including bees and flies (Peitsch et al., 1992; Chittka, 1997). Floral tissue was illuminated with a collimated beam oriented normal to the floral surface, and spectra were collected by a probe positioned at a 45° azimuth, composed of a collimating lens and optical fiber (fiber diameter = 400 microns) connected to the spectrophotometer. We utilized SpectraSuite version 2.0.162 software for capturing spectral data (Ocean Optics, Dunedin, FL, United States). Spectra were collected with an integration time ranging from 50 to 250 ms and a boxcar smoothing width ranging from 3 to 25 nm, with a range of 10–30 average spectral scans (species-specific details of these parameters are included in **Supplementary Table S4**).

In collection of floral spectra, at least one single petal of the floral unit was measured for each flowering species, or in the instance that a single petal was too small to cover the entire sampling area, multiple petals were overlaid to provide enough surface for the spectrometer to collect a reflectance reading (McEwen and Vamosi, 2010). Within each floral unit, if there was a noticeable change in coloration in the human vision color spectrum or morphological component (e.g., petal vs. labellum), reflectance readings were obtained from various portions across the floral unit. We also searched for any change in ultraviolet reflectance range across the floral display area by viewing live spectrometer reflectance output while moving across the floral tissue surface. In total, 24 species of the 55 species were found to have variation in color within a given floral unit. Any noted differences within a floral unit were measured, and these details are included in Supplementary Information for each plant species (**Supplementary Table S5**).

To model floral visitor perception of floral colors under biologically relevant lighting conditions experienced during foraging, we measured solar irradiance at a single location at midday to represent study sites at midday (McLaughlin Natural Reserve Housing Site, Lower Lake, CA, United States: 38°52′23.82″N, 122°25′53.85″W) using a calibrated portable ultraviolet-visible (UV-vis) spectrophotometer (Ocean Optics JAZ, Ocean Optics, Dunedin, FL). Additionally, to represent a typical background against which floral colors were viewed by floral visitors, we measured the green foliage of five plant species occurring in the serpentine seep community (*H. rariflorum*, *Hoita macrostachya*, *Mimulus guttatus*, *Triteleia peduncularis*, and *Toxicoscordion venenosum*) using the same spectrometry techniques as applied to floral color measurement. We averaged these foliage spectra to produce a composite background reflectance spectrum.

Processing of Spectral Data

Within each species, all floral reflectance spectra were averaged to produce representative floral reflectance spectra for each species. If spectral variation within the flowering unit was documented, these reflectances were weighted by the proportion of their representative measured area within a floral unit. This proportion was estimated by searching for distinct changes in UV spectral reflectance along the surface of the flower using the spectrometer (when considering UV internal contrast) or the percent area for each different color in human color perception was estimated by eye. The weighted spectral reflectances were then averaged together to create one reflectance reading for a given species. We chose to use this aggregate reflectance spectra because this represents the information available to floral visitors at typical foraging distances and is thus consistent with the information that might guide flower detection and visitation by insects (Lunau et al., 2006; however, see Garcia et al., 2018).

Floral Visitor Vision Modeling

To identify the predominant floral visitors of the seep metacommunity and choose suitable insect vision models for modeling floral color, we evaluated data from a prior study documenting the insects visiting flowering species in seeps recorded in 2010 (Koski et al., 2015). From over 250 h of visitation observation in these seep communities, Koski et al. (2015) observed 15 functional groups of flower visitors to the seeps. Six of the 15 functional groups were of different groups of bees (large social bees, extra-large social bees, small solitary bees, medium solitary bees, large solitary bee species carrying pollen on legs, and large solitary bee species carrying pollen on body), and four functional groups were flies (Bombyliidae, large-size Syrphidae, small-size Syrphidae, and non-bombyliid/syrphid flies). The majority of flower visitation observed in the seeps were made by these 10 functional groups (Koski et al., 2015). From these findings, we then were able to identify the most common insect visitors, which were primarily bees (Hymenoptera), followed by flies (Diptera) (**Supplementary Table S1**).

To estimate the color appearance of flowers in our seep communities as viewed by their insect visitors, we used receptor noise-limited models of color vision for representative bee (*Apis mellifera*) and fly (*Eristalis tenax*) flower visitors (Vorobyev and Osorio, 1998). We utilized the European honeybee (*A. mellifera*) as our hymenopteran color vision model (Peitsch et al., 1992) because spectral sensitivity data are currently unavailable for the hymenopteran floral visitors endemic to these seep communities (Koski et al., 2015), and spectral sensitivities are largely conserved across Hymenoptera (Briscoe and Chittka, 2001). We also utilized a known syrphid fly color vision model, *E. tenax* (Horridge et al., 1975) because flies, including syrphid flies, were documented as the second-most common flower visitors to the serpentine seep plant community (Koski et al., 2015). Detailed equations of our visual system models are described in **Supplementary Methods S1**.

The European honeybee *A. mellifera* exhibits three color photoreceptor types: ultraviolet (UV), blue (B), and green (G) (Peitsch et al., 1992; Briscoe and Chittka, 2001). The photoreceptor types thought to be generally responsible for color perception in the syrphid fly *E. tenax* are ultraviolet (R7P), violet (R7Y), blue (R8P), and green (R8Y) *sensu* Ohashi et al. (2015). We used the known photoreceptor sensitivities of *A. mellifera* from Peitsch et al. (1992), and we used the known photoreceptor sensitivities of *E. tenax* provided by M. Shrestha and A. G. Dyer (personal communication, Shrestha et al., 2016). For both *A. mellifera* and *E. tenax* vision systems, we modeled the stimulation for all pairwise combinations of flowering species spectra in the regional pool against the collected green background foliage spectra under daylight illumination. This pairwise color disparity estimate between two floral spectra is termed ΔS^t, measured in units of standard deviations of receptor noise between two color stimuli. With larger values of ΔS^t, the two color stimuli are theorized to be more easily distinguishable by the viewer (as calculated using the equations provided in **Supplementary Methods S1**; Vorobyev and Osorio, 1998). The ΔS^t estimates for each pair of floral color spectra were computed with a script using NumPy 1.19.1 (Harris et al., 2020) in Python 3.8.5 (Van Rossum and Drake, 2009) following the methods outlined in Morehouse and Rutowski (2010).

To calculate ΔS^t, photoreceptor noise and relative photoreceptor abundances for *A. mellifera* were incorporated into the model to estimate discriminability following Vorobyev and Osorio (1998). We chose to use a Weber fraction of 0.05 for the *A. mellifera* model, and the relative color photoreceptor abundances of *A. mellifera* were set as 2.125:1:9.375 for UV, B, and G photoreceptor types, respectively (Wakakuwa et al., 2005). Appropriate Weber fraction estimates and relative photoreceptor ratios are not known specifically for *E. tenax*. However, as with *A. mellifera,* we set out Weber fraction to 0.05 and used photoreceptor abundances known generally for flies as 1:2.33:1:2.33 for R7p, R7y, R8p, and R8y, respectively (Earl and Britt, 2006). We then mapped the relative stimulation outputs of each color photoreceptor type (for 55 flowering plant species) into a trichromatic color space for *A. mellifera* using the "ternaryplot" function in the vcd package version 1.4-8 (Meyer et al., 2020) and a tetrachromatic color space for *E. tenax* using the "colspace" function in the pavo package (Maia et al., 2019), both in R version 3.5.3 (R Core Team, 2019).

Trait Community Structure Analyses

We compared the mean color disparity of each seep surveyed in 2013 (observed mean seep ΔS^t) to the mean color disparity of 10,000 randomly assembled communities per observed seep, termed null mean seep ΔS^t. For each of the 14 surveyed (observed) seeps, we generated 10,000 randomly assembled communities of species, with the species richness of the assembled communities limited to the species richness of the observed seeps. For example, if an observed seep in 2013 was found to have six co-flowering species, then it would be compared to 10,000 randomly assembled communities, each composed of six species. Within each of the 10,000 iterations of random community generation, species were drawn from the regional pool of the 55 co-flowering species without replacement. The mean ΔS^t for each randomly assembled community was estimated, and then a grand mean ΔS^t was calculated from all 10,000 communities, thereby creating the null mean seep ΔS^t. This null mean seep ΔS^t was then used for comparison to the observed mean seep ΔS^t for each of the 14 observed seep communities. These randomly assembled communities and estimation of null mean seep ΔS^t values were computed for each pollinator vision system with a script using NumPy 1.19.1 (Harris et al., 2020) in Python 3.8.5 (Van Rossum and Drake, 2009) following the methods outlined in Morehouse and Rutowski (2010).

Analyses of species assemblages restricted to those known to be visited by specifically bees or specifically flies may offer a more functionally relevant and conservative approach for understanding what each flower visitor might experience when foraging in a given seep community. Therefore, we also performed calculations of observed mean ΔS^t and null mean ΔS^t values that were restricted to (a) only the plant species recorded as visited by bees or (b) only the plant species recorded as visited by flies (**Supplementary Table S1**).

To test if (a) the observed mean seep ΔS^t values significantly differed from random expectations (null mean ΔS^t values) and if (b) observed mean seep ΔS^t depended upon seep index, we used analysis of covariance (ANCOVA) (SAS 9.4, PROC GLM). We modeled mean ΔS^t in a community as a function of community type (observed mean seep or null mean seep from 10,000 randomly generated communities) and seep index (the PC1 of observed seep area, plant density, and species richness). These analyses were performed in SAS 9.4 (SAS, 2014). A significant effect of community type suggests that flower color is either overdispersed or underdispersed. A relationship between seep index and trait distribution (mean ΔS^t) is determined by the significance of the interaction term in the model. We ran these analyses for all four scenarios: bee vision system with all plant species, bee vision system with only bee-visited species, fly vision system with all plant species, and fly vision system with only fly-visited species. We also calculated z-scores for each observed community mean ΔS^t in comparison with its null model community mean ΔS^t for all four scenarios. For each ANCOVA, we inspected the normalized residuals of each model

for any spatial autocorrelation using bubble plots, correlograms, variograms, and calculation of Moran's *I*.

Phylogenetic Community Structure Analyses

To evaluate phylogenetic community structure, an ultrametric phylogenetic tree of the regional species pool was constructed including all 55 species for which color was collected (**Supplementary Figure S2**). This was done by using Phylomatic 3.0 and Phylocom 4.2, which incorporated known branch lengths from Wikstrom et al. (2001) using the BLADJ function in Phylocom (2001).

To investigate any patterns of phylogenetic community structure within seep communities that would contextualize evidence for ecological mechanism, we calculated the observed mean phylogenetic distances (MPDs) for each of the seeps surveyed. This observed seep-specific metric was compared against the mean MPD measured from 10,000 random null communities generated for each seep, holding species richness constant but generating communities with random species from the regional (metacommunity) species pool. In effect, this function generates 10,000 random communities and compares the mean pairwise phylogenetic distances for each random community to the observed community.

To test whether (a) observed phylogenetic structure significantly differed from random expectations and whether (b) phylogenetic community structure is related to seep index, we used ANCOVA (SAS, PROC GLM). We modeled MPD as a function of community type (observed vs. null), seep index, and the interaction between community type and seep index in SAS 9.4 (SAS, 2014). A significant difference between observed mean MPD and null community mean MPD values supports overdispersion or underdispersion is present in phylogenetic structure. A significant interaction term in the model will test for significance of any relationship between seep index and phylogenetic overdispersion or underdispersion. For this ANCOVA, we inspected the normalized residuals for any spatial autocorrelation using bubble plots, correlograms, variograms, and calculation of Moran's *I*.

RESULTS

Trait Community Structure

The mean number of plant species recorded per seep was 14 species per seep, with a range of six to 20 species. Of these observed seep communities, an average of approximately 12 plant species per seep was known to be visited by bees (ranging from 5 to 17 species), and an average of approximately seven plant species per seep was known to be visited by flies (with a range from 2 to 13 plant species). For the fly-specific visitor community structure analysis, we chose to exclude the two seep communities that each only had two plant species known to be visited by flies, which were seep SPCL and seep SPCU.

When floral color disparity (mean ΔS^t) among all co-flowering species was modeled using the selected bee color vision model (**Figure 2**) and fly color vision model (**Figure 3**), on average, observed mean seep ΔS^t was overdispersed in observed communities, exhibiting significantly higher color differences compared to null model predictions [bee model, **Figure 4A**: $F_{(3,24)} = 25.53$, $P < 0.0001$; observed mean ΔS^t: 9.698, 95% CI (9.398, 9.998); null mean ΔS^t: 8.660, 95% CI (8.360, 8.960); fly model, **Figure 4B**: $F_{(3,24)} = 34.18$, $P < 0.0001$; observed mean ΔS^t: 25.677, 95% CI (25.019, 26.339); null mean ΔS^t: 23.028, 95% CI (22.366, 23.689)].

The bee model analysis with all plant species revealed a significant interaction between community type (observed vs. null) and seep index [$F_{(3,24)} = 4.35$, $P = 0.0477$], with the observed mean ΔS^t declining with seep index ($t = -2.09$, $P = 0.0477$) but no relationship between the null mean ΔS^t and seep index ($t = -0.03$, $P = 0.9769$) (**Figure 4A**). The fly model with all plant species revealed a marginally significant interaction between community type (observed vs. null) and seep index [$F_{(3,24)} = 4.21$, $P = 0.0511$], with the observed mean ΔS^t exhibiting a marginally significant decline with seep index ($t = -2.05$, $P = 0.0511$) with no relationship between the null mean ΔS^t and seep index ($t = 0.03$, $P = 0.9740$) (**Figure 4B**). No spatial structure was found in the model residuals for bee nor fly models with all plant species (bee model: Moran's $I = -0.1075$, $P = 0.7485$; fly model: Moran's $I = 0.0482$, $P = 0.1869$).

For the model that restricted the plant community to plant species known to be visited by bees, observed mean ΔS^t values calculated with the bee color vision model were significantly overdispersed compared to null model predictions [**Figure 4C**; $F_{(3,24)} = 48.50$, $P < 0.0001$; observed mean ΔS^t: 10.180, 95% CI (9.926, 10.434); null mean ΔS^t: 8.968, 95% CI (8.714, 9.222)]. Additionally, there was a significant interaction between mean

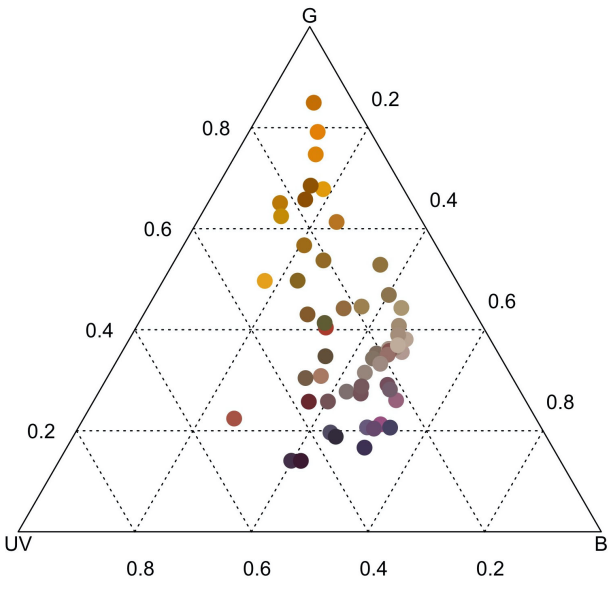


FIGURE 2 | Bee color space depicting the differential stimulation of color photoreceptor types of *Apis mellifera* with all 55 co-flowering species from serpentine seep community. Color of circles represents human-perceived color of flowering structure for each species.

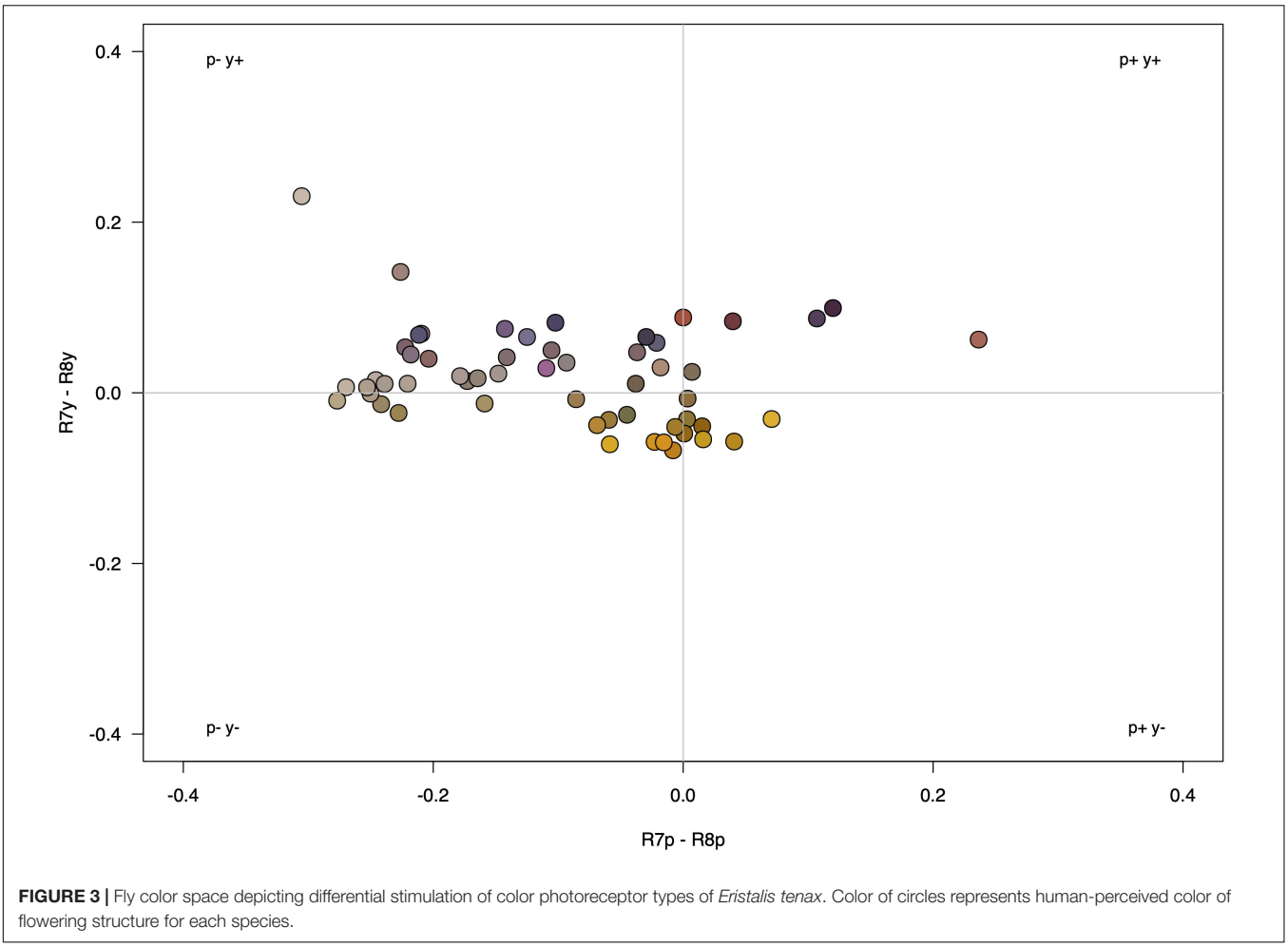


FIGURE 3 | Fly color space depicting differential stimulation of color photoreceptor types of *Eristalis tenax*. Color of circles represents human-perceived color of flowering structure for each species.

community type (observed vs. null) and seep index for the bee-visited community [$F_{(3,24)}$ = 7.14, P = 0.0133], and this interaction was driven by a decline in the observed mean ΔS^t with seep index (t = −2.67, P = 0.0133) but not by the null mean ΔS^t (t = 0.01, P = 0.9917) (**Figure 4C**).

For the model that restricted the plant community to plant species known to be visited by flies, mean ΔS^t values calculated with the fly color vision model were significantly overdispersed compared to null model predictions [**Figure 4D**; $F_{(3,20)}$ = 42.36, $P < 0.0001$; observed mean ΔS^t: 28.105, 95% CI (27.260, 28.950); null mean ΔS^t: 24.685, 95% CI (23.840, 25.530)]. There was also a significant interaction between community type (observed vs. null) and seep index for the fly-visited plant community (observed vs. random $F_{(3,20)}$ = 6.99, P = 0.0156), and this interaction was again driven by a decline in the observed mean ΔS^t with seep index (t = −2.64, P = 0.0156) but not the null mean ΔS^t (t = 0.01, P = 0.9918) (**Figure 4D**). No spatial structure was found in the model residuals for bee nor fly models restricted specifically by known visitation (bee-specific model: Moran's I = 0.0108, P = 0.3431; fly-specific model: Moran's I = −0.1290, P = 0.7056).

All z-scores for each observed mean seep ΔS^t compared to null mean seep ΔS^t are found in **Supplementary Table S6** (bee model with all plant species), S7 (fly model with all plant species), S8 (bee model with bee-visited plant species), and S9 (fly model with fly-visited plant species).

Phylogenetic Community Structure

There was no phylogenetic community structure detected in the observed seep communities, and there was no relationship between seep index and phylogenetic relatedness [$F_{(3,24)}$ = 1.91, P = 0.1542] (**Figure 5**). Seep-specific z-scores are found in **Supplementary Table S10**. No spatial structure was found in the model residuals (Moran's I = −0.0781, P = 0.9898).

DISCUSSION

Observed assemblages of flower color in serpentine seep communities are overdispersed compared to random assemblages when viewing floral colors through models of both bee vision and fly vision. Flower color overdispersion was particularly strong when evaluating the two subsets of plants documented to be visited by bees and flies. Distinctiveness in floral color perception of a given species relative to other co-flowering community members could aid in recognition

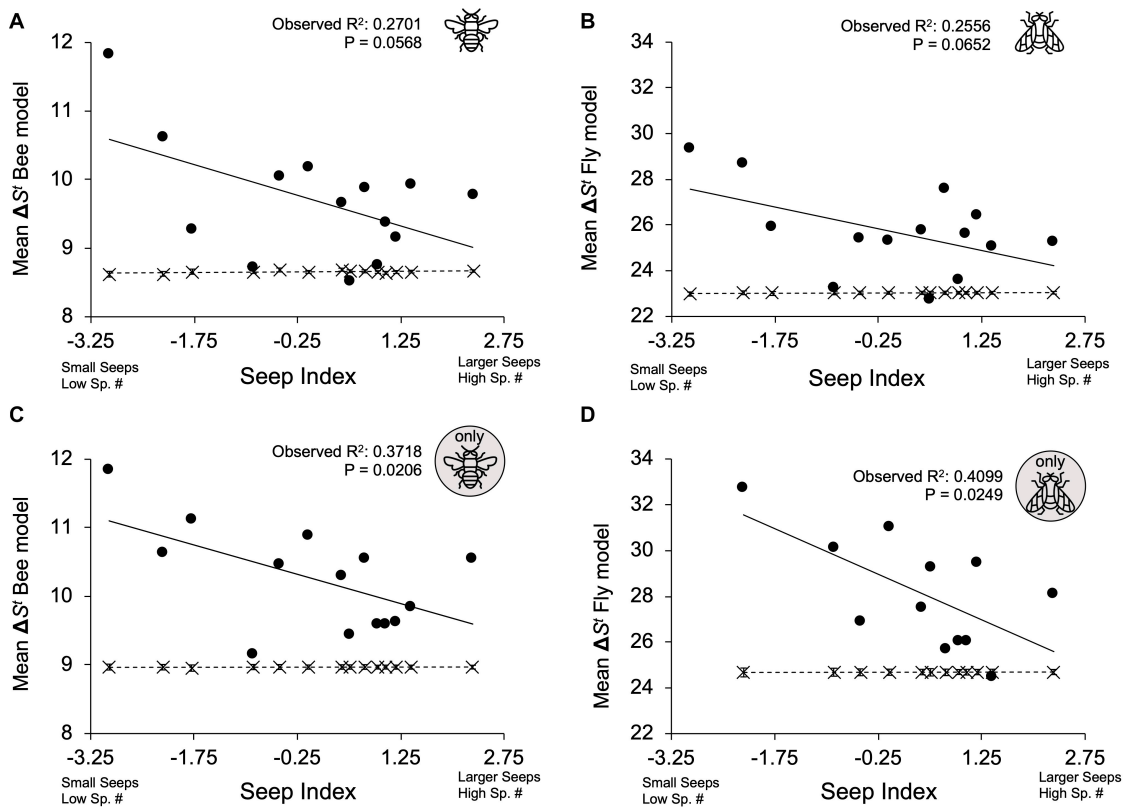


FIGURE 4 | Comparison of observed and null mean ΔS^t values across seep index. Observed seep values are represented by black circles (●) and solid lines; randomly generated null communities are represented by "X" markers and dashed lines. Low seep index values refer to small seeps with low species richness, high seep index values correspond to large seeps with high species richness. Error bars present on null mean ΔS^t values represent 95% confidence intervals. **(A)** All co-flowering species modeled through bee vision system. **(B)** All co-flowering species modeled through fly vision system. **(C)** Model restricted to bee-visited plant species. **(D)** Model restricted to fly-visited plant species.

by pollinators, increase pollinator visitation rates, and support pollinator fidelity (McEwen and Vamosi, 2010; Muchhala et al., 2014). In particular, in the absence of phylogenetic structure, the overdispersion of a functional trait may indicate pollinator-mediated competition (Sargent and Ackerly, 2008). Our study did not evaluate species-specific pollination efficiency by floral visitors but instead considered floral appearance to presumed pollinators. If our assumption of visitors as pollinators holds, then our data support that the observed trait overdispersion is likely the result of ecological mechanisms rather than phylogenetic sorting because we failed to find a nonrandom phylogenetic community structure (**Figure 5**). However, our study did not evaluate instances of character displacement, which could also drive floral trait divergence by natural selection (Sargent and Ackerly, 2008).

In addition to competition for pollinator visits, another mechanism that could produce such a pattern of competitive exclusion and maintain overdispersion in visitor-perceived color is the avoidance of interspecific pollen transfer. Interspecific pollen transfer has been found to negatively impact plant reproductive success, including seed production, when pollinators are shared among plant species (Feinsinger et al., 1988; Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013), with interspecific pollen transfer involving both the loss of conspecific pollen transfer and the deposition of heterospecific pollen (Wilcock and Neiland, 2002; Mitchell et al., 2009). If community membership is structured to maximize pollinator-perceived color disparity, then plant coexistence and persistence can occur with limited negative impacts due to high pollinator fidelity and pollinator recognition (Chittka, 1997; McEwen and Vamosi, 2010). However, for any species immigrating into a community for which it overlaps with already established species in trait space, asymmetrical competition might occur through heterospecific pollen deposition and reduced conspecific pollen transfer (Runquist and Stanton, 2013). However, these predictions should be further experimentally evaluated in a community context, as interactive effects are known to mediate the magnitude of detrimental effects of heterospecific pollen transfer and deposition (Arceo-Gómez and Ashman, 2011; Arceo-Gómez et al., 2019).

Overdispersion of visitor-perceived color disparity was greater at smaller seeps with fewer species (**Figures 4A–D**). Competition may be more intense in small communities with fewer species because of reduced overall visitation by pollinators (Sargent and Ackerly, 2008). When shared pollinators do visit, there

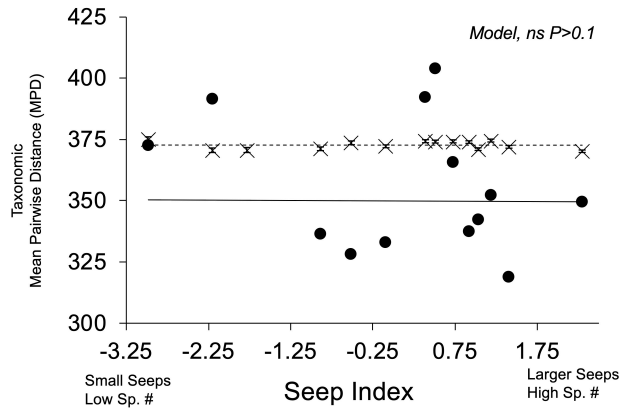


FIGURE 5 | Comparison of observed and null mean phylogenetic distances (MPDs) across seep index. Observed seep values are represented by black circles (●) and solid line; randomly generated null communities are represented by "X" markers and dashed line. Error bars present on null MPD community values represent 95% confidence intervals.

would be the risk of heterospecific pollen transfer for more similarly perceived co-flowering species as described previously. This overdispersion of color disparity decreased with greater seep habitat area and species richness (**Figures 4A–D**), which could suggest that competitive exclusion is a dominant ecological mechanism structuring flower color disparity in smaller, less species-rich seep communities, but this signature of competition may be either less detectable or not occurring in larger habitats. The lack of trait overdispersion in larger communities could be caused by other ecological mechanisms (such as facilitation or habitat filtering) drowning out competitive signals in seeps with a larger habitat area and greater species richness. In particular, this trend toward reduced overdispersion may be due to differences in the shifting dominance from biotic to abiotic mechanisms with increasing species richness and habitat area, regardless of phylogenetic community structure (Arista et al., 2013). Alternatively, lower seep mean ΔS^t could be the result of increased occupancy in color vision trait space exhibited in larger seeps with greater species richness; with larger community assemblages, the more trait values must fit within the same confines of trait space, resulting in higher packing density of color traits. However, further work in a field setting should be done to measure reproductive success of different co-flowering species with experimental manipulations of floral visitor exclusion, such as bees only, flies only, or both visitors in pollination assays.

Other studies have also detected overdispersion of floral color in co-flowering communities using metrics of insect color vision (Muchhala et al., 2014; Makino and Yokoyama, 2015), and our findings are consistent with previous studies finding evidence for competitive exclusion in pollinator color vision space regardless of phylogenetic community structure (Muchhala et al., 2014). Yet other studies have found that floral color is clustered when using pollinator vision models (Kemp et al., 2019) when using other colorimetric analyses independent of pollinator perception (McEwen and Vamosi, 2010). Further, Shrestha et al. (2019) found color assemblages that did not significantly differ from random expectation. Some studies have found evidence for ecological mechanisms at the level of plant clade (Muchhala et al., 2014; Briscoe Runquist et al., 2016) and plant family (Kemp et al., 2019). By studying 14 co-flowering assemblages with varying family associations, habitat availability, and species richness, our findings demonstrate that the detection of patterns such as trait overdispersion may depend on community size and species richness, and therefore, inference of mechanism may vary at these local scales.

Our study is limited by its focus on only one sensory modality involved in insect flower visitor behavior, namely, vision. Consideration of the role of other important sensory elements (e.g., olfactory cues, flower display size, floral reward composition) would allow a more complete understanding of how a community collectively attracts floral visitors and how the resulting interactions shape species presence and persistence in these seep habitats (Primante and Dötterl, 2010; Leonard et al., 2011; Junker et al., 2013; Wei et al., 2020). In addition, behavioral validation of our visual system modeling would help to critically test the role of pollinator–plant interactions in community assemblage implicated in our work. However, due to the relatively high mean ΔS^t community values in observed seeps for both bee and fly models, we predict that behavioral observations would confirm that these estimates of ΔS^t reflect accurate behavioral discrimination among co-flowering species (Fleishman et al., 2016; but see Garcia et al., 2018).

Other factors not considered in this study include an assessment of co-flowering species densities; within co-flowering communities, conspecific and heterospecific plant densities impact their individual reproductive success (Benadi and Pauw, 2018). We were also unable to document the floral color of eight plant species known to occur in this serpentine seep community, in part, because they were infrequently observed, and they were not observed at all in 2013 site surveys. Their rarity may impact the discriminability of the community that we have not been able to measure. We predict that their densities are low in seep areas where they do occur, but how density-dependent plant–pollinator interactions are mediated with changing habitat size and community species richness is an active area of theoretical and empirical study (Mesgaran et al., 2017; Benadi and Pauw, 2018; Bergamo et al., 2020).

In a rapidly changing world threatened with major losses of biodiversity and ecosystem function (Mori et al., 2013), ecologists must seek to elucidate the mechanisms that generate and sustain variation in observed patterns of co-occurring species or trait assemblages (Diamond, 1975). We have shown the evidence of small-scale community structuring in color discriminability in replicated communities, and we have provided evidence that these observed plant communities could be responding to flower visitors as selective forces in community assembly.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the **Supplementary Material**, with the exception of spectral color

data, which can be accessed in an online repository at this link: https://doi.org/10.5061/dryad.v41ns1rtq.

AUTHOR CONTRIBUTIONS

T-LA, GA-G, and KL conceived the study. NM trained KL on color vision modeling and interpretation of model results. GA-G, MK, and KL acquired floral specimens for spectral collection. KL conducted field surveys in 2013, led the writing of the manuscript, statistical analyses, and interpretation of community assembly results. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.602951/full#supplementary-material

Supplementary Figure 1 | Results of principal component analysis. **(A)** Proportion of variation explained by each Principal Component axis, with dashed line representing cumulative proportion, solid line representing proportion. **(B)** Eigenvalues of Principal Component Axes.

Supplementary Figure 2 | Phylogenetic tree of 55 plant species used in study. Circles are colored to represent human vision interpretation of flower color. Branch length reference unit of 10 represents 10 Mya.

Supplementary Table 1 | List of 55 plant species used in study, along with community membership and flower visitor information. Seep sites are ordered by increasing species richness. An X in the "F" Visit column denotes that a focal plant species is visited by fly species, an X in the "B" Visit column denotes that a focal plant species is visited by bee species.

Supplementary Table 2 | Correlation panel for species richness, seep area, and plant density.

Supplementary Table 3 | principal component loadings for species richness, seep area, and plant density.

Supplementary Table 4 | Details of spectra collection parameters for each plant species.

Supplementary Table 5 | Descriptions of noted color differences within the flowering unit of each plant species in the study.

Supplementary Table 6 | Estimated z-scores of each observed seep mean ΔS^t compared to null community mean ΔS^t for bee vision model using all plant species regardless of known insect visitation.

Supplementary Table 7 | Estimated z-scores of each observed seep mean ΔS^t compared to null community mean ΔS^t for fly vision model using all plant species regardless of known insect visitation.

Supplementary Table 8 | Estimated z-scores of each observed seep mean ΔS^t compared to null community mean ΔS^t for bee vision model only using plant species known to be visited by bees.

Supplementary Table 9 | Estimated z-scores of each observed seep mean ΔS^t compared to null community mean ΔS^t for fly vision model only using plant species known to be visited by flies.

Supplementary Table 10 | Estimated z-scores of each observed seep taxonomic Mean Pairwise Distance (MPD) compared to null community taxonomic MPD.

Supplementary Methods 1 | Detail of color vision modeling equations used in methods.

REFERENCES

Ackerly, D. D. (2003). Community assembly, niche conservatism, and adaptive evolution in changing environments. *Int. J. Plant Sci.* 164, S165–S184. doi: 10.1086/368401

Alonso, C., Navarro-Fernández, C. M., Arceo-Gómez, G., Meindl, G. A., Parra-Tabla, V., and Ashman, T.-L. (2013). Among-species differences in pollen quality and quantity limitation: implications for endemics in biodiverse hotspots. *Ann. Bot.* 112, 1461–1469. doi: 10.1093/aob/mct213

Arceo-Gómez, G., and Ashman, T.-L. (2011). Heterospecific pollen deposition: does diversity alter the consequences? *New Phytol.* 192, 738–746. doi: 10.1111/j.1469-8137.2011.03831.x

Arceo-Gómez, G., Kaczorowski, R. L., Patel, C., and Ashman, T.-L. (2019). Interactive effects between donor and recipient species mediate fitness costs of heterospecific pollen receipt in a co-flowering community. *Oecologia* 189, 1041–1047. doi: 10.1007/s00442-019-04379-z

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Ashman, T.-L., and Arceo-Gómez, G. (2013). Toward a predictive understanding of the fitness costs of heterospecific pollen receipt and its importance in co-flowering communities. *Am. J. Bot.* 100, 1061–1070. doi: 10.3732/ajb.1200496

Benadi, G., and Pauw, A. (2018). Frequency dependence of pollinator visitation rates suggests that pollination niches can allow plant species coexistence. *J. Ecol.* 106, 1892–1901. doi: 10.1111/1365-2745.13025

Benitez-Vieyra, S., de Ibarra, N. H., Wertlen, A. M., and Cocucci, A. A. (2007). How to look like a mallow: evidence of floral mimicry between Turneraceae and Malvaceae. *Proc. R. Soc. B.* 274, 2239–2248. doi: 10.1098/rspb.2007.0588

Bergamo, P. J., Susin Streher, N., Traveset, A., Wolowski, M., and Sazima, M. (2020). Pollination outcomes reveal negative density-dependence coupled with interspecific facilitation among plants. *Ecol. Let.* 23, 129–139. doi: 10.1111/ele.13415

Binkenstein, J., Renoult, J. P., and Schaefer, H. M. (2013). Increasing land-use intensity decreases floral colour diversity of plant communities in temperate grasslands. *Oecologia* 173, 461–471. doi: 10.1007/s00442-013-2627-6

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Ann. Rev. Entomol.* 46, 471–510.

Briscoe Runquist, R., Grossenbacher, D., Porter, S., Kay, K., and Smith, J. (2016). Pollinator-mediated assemblage processes in California wildflowers. *J. Evol. Biol.* 29, 1045–1058. doi: 10.1111/jeb.12845

Bruno, J. F., Stachowicz, J. J., and Bertness, M. D. (2003). Inclusion of facilitation into ecological theory. *Trends Ecol. Evol.* 18, 119–125. doi: 10.1016/S0169-5347(02)00045-9

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B* 281:20132862. doi: 10.1098/rspb.2013.2862

Cavender-Bares, J., Keen, A., and Miles, B. (2006). Phylogenetic structure of Floridian plant communities depends on taxonomic and spatial scale. *Ecology* 87, S109–S122.

Chittka, L. (1997). Bee color vision is optimal for coding flower color, but flower colors are not optimal for being coded—Why? *Isr. J. Plant Sci.* 45, 115–127. doi: 10.1080/07929978.1997.10676678

de Jager, M. L., Dreyer, L. L., and Ellis, A. G. (2011). Do pollinators influence the assembly of flower colours within plant communities? *Oecologia* 166, 543–553. doi: 10.1007/s00442-010-1879-7

Diamond, J. M. (1975). "Assembly of species communities," in *Ecology and Evolution of Communities*, eds M. L. Cody and J. M. Diamond (Cambridge, MA: Harvard University Press), 342–444.

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Earl, J. B., and Britt, S. G. (2006). Expression of *Drosophila* rhodopsins during photoreceptor cell differentiation: insights into R7 and R8 cell subtype commitment. *Gene Expr. Patterns* 6, 687–694. doi: 10.1016/j.modgep.2006.01.003

E-Vojtkó, A., Bello, F., Durka, W., Kühn, I., and Götzenberger, L. (2020). The neglected importance of floral traits in trait-based plant community assembly. *J. Veg. Sci.* 31, 529–539. doi: 10.1111/jvs.12877

Fargione, J., Brown, C. S., and Tilman, D. (2003). Community assembly and invasion: an experimental test of neutral versus niche processes. *Proc. Natl. Acad. Sci.* 100, 8916–8920. doi: 10.1073/pnas.1033107100

Feinsinger, P., Busby, W. H., and Tiebout, H. M. (1988). Effects of indiscriminate foraging by tropical hummingbirds on pollination and plant reproductive success: experiments with two tropical treelets (Rubiaceae). *Oecologia* 76, 471–474. doi: 10.1007/bf00377045

Fleishman, L. J., Perez, C. W., Yeo, A. I., Cummings, K. J., Dick, S., and Almonte, E. (2016). Perceptual distance between colored stimuli in the lizard *Anolis sagrei*: comparing visual system models to empirical results. *Behav. Ecol. Sociobiol.* 70, 541–555. doi: 10.1007/s00265-016-2072-8

Freestone, A. L., and Harrison, S. (2006). Regional enrichment of local assemblages is robust to variation in local productivity, abiotic gradients, and heterogeneity. *Ecol. Lett.* 9, 95–102. doi: 10.1111/j.1461-0248.2005.00849.x

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2018). The path to colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Compar. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Ghazoul, J. (2006). Floral diversity and the facilitation of pollination. *J. Ecol.* 94, 295–304. doi: 10.1111/j.1365-2745.2006.01098.x

Gumbert, A., Kunze, J., and Chittka, L. (1999). Floral colour diversity in plant communities, bee colour space and a null model. *Proc. R. Soc. Lond. Ser. B.* 266, 1711–1716. doi: 10.1098/rspb.1999.0836

Harris, C. R., Millman, K. J., van der Walt, S. J., Gommers, R., Virtanen, P., Cournapeau, D., et al. (2020). Array programming with NumPy. *Nature* 585, 357–362. doi: 10.1038/s41586-020-2649-2

Harrison, S., Viers, J. H., and Quinn, J. F. (2000). Climatic and spatial patterns of diversity in the serpentine plants of California. *Div. Distrib* 6, 153–162. doi: 10.1046/j.1472-4642.2000.00082.x

Horridge, G. A., Mimura, K., and Tsukahara, Y. (1975). Fly photoreceptors-II. Spectral and polarized light sensitivity in the drone fly Eristalis. *Proc. R. Soc. Londo. Ser. B. Biol. Sci.* 190, 225–237. doi: 10.1098/rspb.1975.0089

Hubbell, S. P. (2001). *The Unified Neutral Theory of Biodiversity and Biogeography*. Princeton, NJ: Princeton University Press.

Junker, R. R., Blüthgen, N., Brehm, T., Binkenstein, J., Paulus, J., Martin Schaefer, H., et al. (2013). Specialization on traits as basis for the niche—breadth of flower visitors and as structuring mechanism of ecological networks. *Funct. Ecol.* 27, 329–341. doi: 10.1111/1365-2435.12005

Kemp, J. E., Bergh, N. G., Soares, M., and Ellis, A. G. (2019). Dominant pollinators drive non-random community assembly and shared flower colour patterns in daisy communities. *Ann. Bot.* 123, 277–288. doi: 10.1093/aob/mcy126

Koski, M. H., Meindl, G. A., Arceo-Gómez, G., Wolowski, M., LeCroy, K. A., and Ashman, T.-L. (2015). Plant–flower visitor networks in a serpentine metacommunity: assessing traits associated with keystone plant species. *Arthr. Plant Interact.* 9, 9–21. doi: 10.1007/s11829-014-9353-9

Kraft, N. J. B., Cornwell, W. K., Webb, C. O., and Ackerly, D. D. (2007). Trait evolution, community assembly, and the phylogenetic structure of ecological communities. *Am. Natur.* 170, 271–283. doi: 10.1086/519400

Kruckeberg, A. (1984). The flora on California's serpentine. *Fremontia (U.S.A.)* 11, 3–10.

Leonard, A. S., Dornhaus, A., and Papaj, D. R. (2011). Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J. Exp. Biol.* 214, 113–121. doi: 10.1242/jeb.047407

Leonard, A. S., and Masek, P. (2014). Multisensory integration of colors and scents: insights from bees and flowers. *J. Compar. Physiol. A* 200, 463–474. doi: 10.1007/s00359-014-0904-4

Lunau, K., Fieselmann, G., Heuschen, B., and van de Loo, A. (2006). Visual targeting of components of floral colour patterns in flower-naïve bumblebees (*Bombus terrestris*; Apidae). *Naturwissenschaften* 93, 325–328. doi: 10.1007/s00114-006-0105-2

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210X.13174

Makino, T. T., and Yokoyama, J. (2015). Nonrandom composition of flower colors in a plant community: mutually different co-flowering natives and disturbance by aliens. *PLoS One* 10:e0143443. doi: 10.1371/journal.pone.0143443

McEwen, J. R., and Vamosi, J. C. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proc. R. Soc. B* 277, 2957–2965. doi: 10.1098/rspb.2010.0501

Mesgaran, M. B., Bouhours, J., Lewis, M. A., and Cousens, R. D. (2017). How to be a good neighbour: facilitation and competition between two co-flowering species. *J. Theoret. Biol.* 422, 72–83. doi: 10.1016/j.jtbi.2017.04.011

Meyer, D., Zeileis, A., and Hornik, K. (2020). *vcd: Visualizing Categorical Data*. R package version 1.4-8.

Mitchell, R. J., Flanagan, R. J., Brown, B. J., Waser, N. M., and Karron, J. D. (2009). New frontiers in competition for pollination. *Ann. Bot.* 103, 1403–1413. doi: 10.1093/aob/mcp062

Moeller, D. A. (2004). Facilitative interactions among plants via shared pollinators. *Ecology* 85, 3289–3301. doi: 10.1890/03-0810

Morales, C. L., and Traveset, A. (2008). Interspecific pollen transfer: magnitude, prevalence and consequences for plant fitness. *Crit. Rev. Plant Sci.* 27, 221–238. doi: 10.1080/07352680802205631

Morehouse, N. I., and Rutowski, R. L. (2010). In the eyes of the beholders: female choice and avian predation risk associated with an exaggerated male butterfly color. *Am. Natur.* 176, 768–784. doi: 10.1086/657043

Mori, A. S., Furukawa, T., and Sasaki, T. (2013). Response diversity determines the resilience of ecosystems to environmental change: response diversity and ecosystem resilience. *Biol. Rev.* 88, 349–364. doi: 10.1111/brv.12004

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in andean solanaceae: competition for pollination shapes flower color variation. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral colour change in the eyes of pollinators: testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144–1155. doi: 10.1111/1365-2435.12420

Ollerton, J., Winfree, R., and Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos* 120, 321–326. doi: 10.1111/j.1600-0706.2010.18644.x

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Compar. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Primante, C., and Dötterl, S. (2010). A syrphid fly uses olfactory cues to find a non-yellow flower. *J. Chem. Ecol.* 36, 1207–1210. doi: 10.1007/s10886-010-9871-6

R Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rathcke, B. (1983). "Competition and facilitation among plants for pollination," in *Pollination Biology*, ed. L. Real (New York, NY: Academic Press), 305–329. doi: 10.1016/b978-0-12-583980-8.50019-3

Runquist, R. B., and Stanton, M. L. (2013). Asymmetric and frequency-dependent pollinator-mediated interactions may influence competitive displacement in two vernal pool plants. *Ecol. Lett.* 16, 183–190. doi: 10.1111/ele.12026

Sargent, R. D., and Ackerly, D. D. (2008). Plant–pollinator interactions and the assembly of plant communities. *Trends Ecol. Evol.* 23, 123–130. doi: 10.1016/j.tree.2007.11.003

SAS (2014). *Cary*. North Carolina: SAS Institute, Inc.

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019). Floral colour structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol. J.* 18, 842–850. doi: 10.1111/plb.12456

van der Niet, T., and Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol. Evol.* 27, 353–361. doi: 10.1016/j.tree.2012.02.002

Van Rossum, G., and Drake, F. L. (2009). *Python 3 Reference Manual*. Scotts Valley, CA: CreateSpace.

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. B* 265, 351–358. doi: 10.1098/rspb.1998.0302

Wakakuwa, M., Kurasawa, M., Giurfa, M., and Arikawa, K. (2005). Spectral heterogeneity of honeybee ommatidia. *Naturwissenschaften* 92, 464–467. doi: 10.1007/s00114-005-0018-5

Waser, N. M. (1986). Flower constancy: definition, cause, and measurement. *Am. Natur.* 127, 593–603. doi: 10.1086/284507

Webb, C. O. (2000). Exploring the phylogenetic structure of ecological communities: an example for rain forest trees. *Am. Natur.* 156, 145–155. doi: 10.2307/3079215

Webb, C. O., Ackerly, D. D., McPeek, M. A., and Donoghue, M. J. (2002). Phylogenies and community ecology. *Ann. Rev. Ecol. Syst.* 33, 475–505. doi: 10.2307/3069271

Wei, N., Kaczorowski, R. L., Arceo-Gómez, G., O'Neill, E. M., Hayes, R. A., and Ashman, T.-L. (2020). Pollinator niche partitioning and asymmetric facilitation contribute to the maintenance of diversity. *Ecology* doi: 10.1101/2020.03.02.974022

Wikstrom, N., Savolainen, V., and Chase, M. W. (2001). Evolution of the angiosperms: calibrating the family tree. *Proc. R. Soc. B* 268, 2211–2220. doi: 10.1098/rspb.2001.1782

Wilcock, C., and Neiland, R. (2002). Pollination failure in plants: why it happens and when it matters. *Trends Plant Sci.* 7, 270–277. doi: 10.1016/s1360-1385(02)02258-6

Wolf, A., Brodmann, P. A., and Harrison, S. (1999). Distribution of the rare serpentine sunflower, Helianthus exilis (Asteraceae): the roles of habitat availability, dispersal limitation and species interactions. *Oikos* 84, 69–76. doi: 10.2307/3546867

Wolowski, M., Carvalheiro, L. G., and Freitas, L. (2017). Influence of plant–pollinator interactions on the assembly of plant and hummingbird communities. *J. Ecol.* 105, 332–344. doi: 10.1111/1365-2745.12684

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Phenotypic Selection on Flower Color and Floral Display Size by Three Bee Species

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Plants exhibit a wide array of floral forms and pollinators can act as agent of selection on floral traits. Two trends have emerged from recent reviews of pollinator-mediated selection in plants. First, pollinator-mediated selection on plant-level attractants such as floral display size is stronger than on flower-level attractant such as flower color. Second, when comparing plant species, distinct pollinators can exert different selection patterns on floral traits. In addition, many plant species are visited by a diverse array of pollinators but very few studies have examined selection by distinct pollinators. In the current study, we examined phenotypic selection on flower color and floral display size by three distinct bee species, the European honey bee, *Apis mellifera*, the common eastern bumble bee, *Bombus impatiens*, and the alfalfa leafcutting bee, *Megachile rotundata,* foraging on *Medicago sativa.* To estimate phenotypic selection by each bee species and for all bees combined simultaneously and on the same group of plants, we introduce a new method that combines pollinator visitation data to seed set and floral trait measurements data typical of phenotypic selection study. When comparing floral traits, all bee species selected on the number of racemes per stem and the number of stems per plant, two components of floral display size. However, only leafcutting bees selected on hue or flower color and only bumble bees selected on chroma or darkness of flowers. Selection on chroma occurred via correlational selection between chroma and number of open flowers per raceme and we examine how correlational selection may facilitate the evolution of flower color in plant populations. When comparing bee species, the three bee species exerted similar selection pattern on some floral traits but different patterns on other floral traits and differences in selection patterns were observed between flower-level and plant-level attractants. The trends detected were consistent with previous studies and we advocate the approach introduced here for future studies examining the impact of distinct pollinators on floral trait evolution.

Keywords: bumble bee, correlational selection, floral display size, flower color, honeybee, leafcutting bee, phenotypic selection

INTRODUCTION

Plants exhibit a high level of floral trait diversity. Flower size, flower color, flower shape, and various aspects of floral display size can vary among plants in a population, among populations of a plant species or among plant species (Brunet, 2009; Dart et al., 2012). The role of pollinators in shaping such floral diversity has been of great interest to evolutionary biologists (Galen, 1996; Fishman and Willis, 2008; Harder and Johnson, 2009; Sletvold et al., 2017). In the last three decades, the attention has focused on identifying the role of pollinators, as opposed to other biotic or to abiotic factors, as agent of selection on floral traits (Strauss and Whittall, 2006; Parachnowitsch and Kessler, 2010; Caruso et al., 2019). The two literature reviews of phenotypic selection in plants have indicated that selection on floral traits by pollinators tend to be greater than by herbivores (Parachnowitsch and Kessler, 2010; Caruso et al., 2019) but can be of similar strength as selection by abiotic factors (Caruso et al., 2019).

To isolate the impact of pollinators on selection of floral traits, it has been suggested to measure phenotypic selection in two groups of plants, one group of hand-pollinated and one group of open-pollinated plants (Fishman and Willis, 2008; Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010; Sletvold et al., 2017). Selection gradients are estimated for each group and the difference in the selection gradients between the hand-pollinated and the open-pollinated treatments is attributed to pollinator-mediated selection on the floral traits of interest. When concentrating on directional selection for studies that compared hand-pollinated and open-pollinated treatments, two patterns emerged. First, pollinators differentially selected on distinct categories of floral traits. Selection was strongest on floral traits associated with pollinator efficiency such as the length of the corolla tube, followed by plant level traits associated with pollinator attraction such as floral display size and finally selection was weakest for flower level traits associated with pollinator attraction such as flower size and flower color (Caruso et al., 2019). Second, distinct pollinators had different impacts on the selection of floral traits and, among plant species, long-tongue flies or birds tended to exert the strongest selection on floral traits and Lepidoptera the weakest (Caruso et al., 2019).

Few studies have compared selection by distinct pollinators within a plant species (Sahli and Conner, 2011; Kulbaba and Worley, 2012, 2013). Conflicting selection among pollinators was identified for some floral traits while for other traits distinct pollinators exerted similar patterns of selection (Sahli and Conner, 2011). For example, in *Polemonium brandegeei*, hummingbirds selected for stigmas exserted beyond the anthers and for longer and wider corolla tubes while hawkmoths selected for stigmas recessed below the anthers and for narrower corolla tubes (Kulbaba and Worley, 2012, 2013). These studies examined each pollinator separately and on different sets of plants (Kulbaba and Worley, 2013) or examined pollinators separately and combined in cages (Sahli and Conner, 2011). But plants in natural populations are differentially visited by distinct pollinators whose abundance and efficiency vary and it would be useful to quantify the impact of the major pollinators on the floral traits of interest simultaneously and on the same group of plants.

Pollinator visitation has been used as a proxy for reproductive success in some phenotypic selection studies (Campbell et al., 1997; Zhao and Wang, 2015). Here, we propose to combine pollinator observations with measurements of seed set and floral traits of plants to examine phenotypic selection on floral traits by distinct pollinators. Each plant in the population is expected to receive differential visits by the distinct pollinator species and a different proportion of its flowers will be visited by each species. Such proportions can be used to differentially attribute seeds set on a plant to the distinct pollinators. In addition, data on pollinator efficiency can be combined with flower visits data to proportionally attribute seeds to each pollinator species. Relative fitness (RF) and phenotypic selection can then be measured within each bee species. Phenotypic selection by all pollinators combined can be measured using total seed set per plant to calculate RF. This approach is developed here to illustrate how phenotypic selection by all pollinators combined can be differentially attributed to each pollinator species as we study phenotypic selection on flower color and floral display size by three bee species in *Medicago sativa*. We determine whether selection by pollinators is stronger for plant-level attractants like floral display size than for flower-level attractants such as flower color (Caruso et al., 2019). We also examine whether the three bee species exert similar or different patterns of selection on these floral traits and how this translates into the overall pattern of selection on the plants. Measuring selection on floral traits by different bee species on the same group of plants provides a more realistic depiction of pollinator-mediated selection on floral traits in plant populations.

MATERIALS AND METHODS

Study Species

Medicago sativa L. is an open-pollinated perennial legume that requires bees for seed production. Flowers are clustered into racemes and plants exhibit variation in the number of open flowers per raceme, number of racemes per stem (inflorescence), and number of stems per plant and flower color can also vary, ranging from shades of purple, to white, to yellow (Bauer et al., 2017).

Medicago sativa flowers require tripping for pollination, where pollinators apply pressure to the keel of the flowers which releases its anthers and stigmas. Flowers remain open following tripping but there is little evidence of further pollen deposition by pollinators on already tripped flowers (J. Brunet, pers. obs.). The tripping rate, the proportion of visited flowers that are tripped by a pollinator, varies among bee species (Cane, 2009; Brunet and Stewart, 2010; Pitts-Singer and Cane, 2011). Typically, alfalfa leafcutting bees have the highest tripping rate, followed by bumble bees and finally honey bees (Pitts-Singer and Cane, 2011; Brunet et al., 2019). Honey bees (*Apis mellifera*) and alfalfa leafcutting bees (*Megachile rotundata*) are used as managed pollinators in alfalfa seed production fields. In addition, many wild bee species, including the common eastern bumble bee (*Bombus impatiens*), are known to visit and effectively pollinate alfalfa (Brookes et al., 1994; Brunet and Stewart, 2010).

Experimental Set Up

Five patches of *M. sativa* with 81 plants per patch, initially planted 0.3 m apart, were set up in a linear arrangement at the West Madison Agricultural Research Station in Madison, WI. One bumble bee hive was set up at the center edge, one honey bee hive 30 m away, and a leafcutting bee domicile was set up at the northwest corner facing southeast. About 1.2 lbs of leafcutting bees were released prior to *M. sativa* peak bloom. Floral trait and fitness measurements were obtained from all flowering plants in two center patches where each plant was numbered.

Floral Traits

The floral traits examined in this study included components of floral display size and flower color. For floral display size, we recorded the number of stems per plant, racemes per stem and open flowers per raceme. For each flowering plant, we counted the number of stems and the number of racemes per stem on ten randomly selected stems or on all stems if a plant had fewer than ten stems. The number of open flowers per raceme was recorded on ten randomly selected racemes per plant. The average number of racemes per stem and open flowers per raceme were tabulated for each plant.

Flower color was determined from spectral measurements of the banner petal for three flowers per plant using the USB 4000 spectrophotometer (Ocean Optics, Orlando, Fl., 350–1,000 nm). Reflectance data were analyzed using Spectra Suite v.10.7.1 software (Ocean Optics). Flowers of *M. sativa* do not reflect in the UV range (Bauer et al., 2017) and spectral measurements were taken in the visible light range (400–700 nm). We used equations from Endler (1990) as modified by Smith (2014) to calculate three components of flower color: chroma (darkness or saturation), hue (color), and reflectivity (brightness). Details of these calculations can be found in Bauer et al. (2017). A plant value represented the average of the three flowers.

An alternative would have been to use a hexagon color vision model, a method that considers bee photoreceptors when quantifying color (Chittka, 1992; Chittka and Kevan, 2005). We have used such models to examine how flower color affected the choice of plants by bees (Bauer et al., 2017). However, in this study, while bees may be doing the selection, they are selecting on the plant traits and not on their perception of those traits. We thus chose hue, chroma and receptivity to describe flower color. While the best method to quantify flower color when pollinators are selecting on the trait may deserve further attention, such discussion is beyond the scope of this study.

Female Reproductive Success

We used the total number of seeds produced per plant as a measure of female reproductive success. On each plant, on ten randomly selected stems or all stems if a plant had fewer than ten stems, we counted the number of pods per stem. A pod is a fruit developing from one flower on a raceme. We collected ten randomly selected fruiting racemes per plant and placed each one in an individually marked paper coin envelope. In the laboratory, the number of pods per raceme were recorded and pods were shredded to obtain the number of seeds per raceme. For each plant, we obtained the average number of mature seeds per pod per raceme and, using the 10 fruiting racemes per plant, we calculated the average number of mature seeds per pod on a plant. To obtain the total number of seeds set per plant, we multiplied the average number of pods per stem by the average number of seeds per pod and multiplied this value by the number of stems produced on a plant.

Proportion of Seeds Attributable to Each Bee Species

To estimate the proportion of seeds on each plant attributable to a given bee species, we used available data on the number of flower visits to a plant by each of the three bee species. Pollinator visitation data were collected on these plants during a two-week observation period at peak bloom for *M. sativa* the year of the study (Bauer et al., 2017). To determine the number of pollinator visits to a plant, we followed bees in a patch and two observers recorded each plant visited by a bee, the number of racemes visited on a plant and flowers visited per raceme on each plant until the bee left the patch or was lost to the observers. This provided floral visits by at least one of the three pollinators for most plants in the two patches (**Supplementary Data**). The pattern of visitation in the patches was typical for the major bee species visiting *M. sativa* throughout its flowering period. To attribute the number of seeds to a bee species based on the number of flower visits, for each plant, we multiplied the proportion of flowers visited by each of the three bee species by the number of seeds set on that plant. This approach links, for each plant, the pollinator visitation data to its seed set during that period, as seeds were collected about four weeks following pollinator observations, the period it takes for fruits and seeds to mature in this plant species.

The number of flowers visited by a bee species is a useful measure of pollinator visits, but to better link floral visits to seed set we also integrated the tripping rate of a bee species to the floral visitation data. In *M. sativa*, flowers must be tripped before they can produce seeds and tripping rate varies among bee species (Cane, 2009; Pitts-Singer and Cane, 2011). We obtained a second measure of pollinator visits which combined floral visits with the tripping rate of a bee species. Previous observations in the area indicated a tripping rate of 55% for bumble bee, 25% for honey bee (Brunet and Stewart, 2010) and 80% for leafcutting bee under warm temperatures typical of alfalfa seed-production fields (Brunet et al., 2019). For each plant, the number of flowers visited by a bee species on that plant was multiplied by the bee species specific tripping rate. We call this measure the number of flowers tripped by a bee species. For each plant, we calculated the number of tripped flowers by each bee species and the proportion of flowers tripped by each bee species. We multiplied these proportions by the number of seeds set on the plant to assign seeds to each of the three bee species based on the number of tripped flowers.

Plant Relative Fitness

Plant relative fitness (RF) was estimated by dividing the absolute fitness of a plant by the mean absolute fitness of the group

of plants under consideration (Lande and Arnold, 1983). The absolute fitness of a plant was quantified as the number of seeds set on a plant. RF was obtained for all plants for which floral trait measurements were available (N = 153). We calculated RF of a plant over all bees, based on the total number of seeds it produced, and within each bee species. Within a bee species, RF was the number of seeds on a plant attributable to a given bee species, based either on the proportion of flowers visited or the proportion of flowers tripped by a specific bee species, divided by the mean for that bee species. Using this approach, the mean RF was 1.0 within each bee species and potential differences in seed production across pollinators were removed. We also calculated the opportunity for selection for overall RF and for RF by bee species based on proportion of visits or proportion of tripped flowers. Opportunity for selection was measured as the variance in RF.

Phenotypic Selection

To measure phenotypic selection, we examined the relationship between the trait value of a plant and its RF (Lande and Arnold, 1983). Each floral trait examined was scaled such that its mean was 0 and its variance was 1: (trait value – trait mean)/trait standard deviation. We performed phenotypic selection analyses on the number of stems per plant, the number of racemes per stem, the number of open flowers per raceme, and hue, chroma and reflectivity. We first performed phenotypic selection analyses using RF calculated over the total seed set of a plant. We also examined phenotypic selection within each bee species, where RF was calculated as explained earlier, either based on proportion of flowers visited or proportion of flowers tripped by a bee species on each plant. RF was relativized and traits standardized within each bee species which eliminated any potential differences in traits or fitness across bee species. The number of plants was similar for overall fitness and within each bee species and represented plants with floral traits and seed set data (N = 153). The number of plants that received no visits by a specific bee species did vary, with a greater number of plants not visited by leafcutting bee (N = 107 plants), followed by honey bee (N = 46) and last bumble bee (N = 16).

We used regression analyses, examining linear and non-linear regressions, to estimate various selection parameters, following the methods suggested by Lande and Arnold (1983). Untransformed variables were used to obtain the values of the selection coefficients. To obtain the statistical significance of the selection coefficients, RF values were log transformed in order to improve the model's residuals. This procedure was followed because selection coefficients are not known to be affected by a poorly fit model while the probability values are (Lande and Arnold, 1983; Mitchell-Olds and Shaw, 1987; Brodie and Janzen, 1996). In addition, due to the large number of zeros, the model's residuals for leafcutting bee still indicated a poor fit to the data after transforming RF. We therefore used bootstrapping to estimate the 95% confidence intervals around the selection coefficients and determine whether they were statistically significant (Davison and Hinkley, 1997). We used bootstrapping for all cases for comparison purpose. We performed 1,000 bootstraps using the bootstrap function in the package "boot" (Canty and Ripley, 2020) in R (version 3.6.1).

For directional selection, we estimated the selection differential (S_i), which represents the change in the population mean of trait (i) after selection (Arnold and Wade, 1984). The selection differential can be obtained from the slope of a linear regression between the standardized value of a trait and the corresponding plant RF. This coefficient includes both direct and indirect selection and multiple regression analyses were performed to isolate direct selection. The partial regression coefficient for a trait represents the selection gradient (βi) for that trait (i) and illustrates direct selection on a trait after removing indirect selection from all other traits present in the analysis. When traits are correlated, a trait that appears to respond to selection may simply be correlated to the trait under selection, hence the need to isolate direct selection. The coefficients S or β both represent directional selection and a positive value indicates that the phenotypic mean of a trait (i) increases under selection while it decreases when values of Si or βi are negative.

Because selection can also be non-linear and work on the shape of the trait distribution, we first added a quadratic term to the single regression and obtained the non-linear (quadratic) selection differential C_{ii} (**Table 1**), where C_{22} illustrates the non-linear (quadratic) term of the single regression. We then performed multiple regressions with linear, quadratic and cross product terms to obtain the non-linear or quadratic selection gradient γ_{ii}, represented by the partial regression coefficient for the quadratic term, and to detect correlational selection γ_{ij} using the partial regression coefficient for the cross product terms (**Table 1**; Brodie, 1992; Roff and Fairbairn, 2012). The quadratic coefficient gradients were estimated as double the quadratic regression coefficients (Stinchcombe et al., 2008; Sahli and Conner, 2011).

We graphically illustrated the statistically significant cross product terms representing correlational selection gradients using the function "persp" in R (R Core Team, 2019). To represent the non-linear selection for the statistically significant quadratic selection gradients we used generalized additive models (GAMs) using the "mgcv" package in R (Wood, 2011). These models automatically fit a spline regression (Wood, 2011). Results from the GAMs were plotted using "ggplot2"(Wickham, 2016) and "gridExtra"(Auguie, 2017).

Besides using regression analyses, we also examined the distributional selection gradient on the floral traits (Henshaw and Zemel, 2017). This measures total selection on a trait and can be broken down into a directional component (dD) illustrating selection on a trait mean and a non-directional component (dN) that reflects selection on the shape of the trait distribution. This approach permits estimation of the general selection differential (S) and selection gradients (β). We used the R code available from

TABLE 1 | Selection parameters obtained based on different regression analyses using plant relative fitness and standardized floral traits.

Model	Single regression		Multiple regression	
Linear	S	Selection differential; slope; both direct and indirect selection	β	Selection gradient; partial regression coefficient; direct selection
Non-linear (linear and quadratic terms)	C_{ii}	Non-linear or quadratic selection differential is C_{22}		
Non-linear (linear, quadratic and cross product terms)			γ_{ii}	Non-linear or quadratic selection gradient; partial regression coefficient of quadratic term
Non-linear (linear, quadratic and cross product terms)			γ_{ij}	Correlational selection gradient; Partial regression coefficient of cross product term

The subscript i indicates trait(i) and j indicates a separate trait.

TABLE 2 | Phenotypic selection with relative fitness calculated over all bees (*N* = 153) using either the regression model with log transformed relative fitness or bootstrapping to determine the statistical significance of the selection coefficients.

Statistical Method		Log transformed		Bootstrap		
Trait	**Selection coefficient**	**Estimate**	***P***	**Estimate**	**Lower 95%**	**Upper 95%**
Racemes per Stem	S	0.297	<0.0001	0.297	0.196	0.422
Racemes per Stem	β	0.199	<0.0001	0.199	0.106	0.308
Racemes per Stem	C_{22}	−0.139	0.004	−0.139	−0.272	−0.055
Stems per plant	S	0.612	<0.0001	0.612	0.419	0.801
Stems per plant	β	0.578	<0.0001	0.578	0.383	0.764
Open Flowers per raceme	γ_{FlrChr}	0.370	0.022	0.370	NS	NS

*The methodology followed to obtain the different selection coefficients is summarized in **Table 1**. The letter P stands for probability and NS for not statistically significant. Flr stands for open flowers per raceme and Chr for flower chroma.*

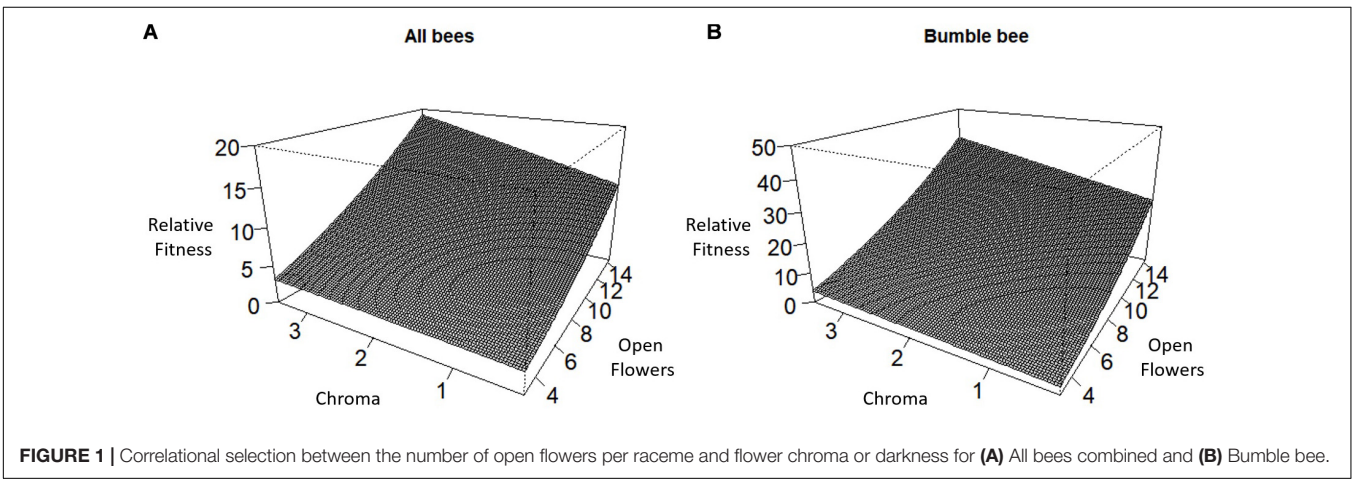


FIGURE 1 | Correlational selection between the number of open flowers per raceme and flower chroma or darkness for **(A)** All bees combined and **(B)** Bumble bee.

Github[1] to run distributional selection differential analyses on our data following Henshaw and Zemel, 2017.

RESULTS

Floral Traits, Bee Visits, and Plant Relative Fitness

Data on floral traits and seed set were recorded for 153 plants. We observed 8,727 flower visits on these plants, with 4,570 (52.3%) visits by bumble bees, 3,925 (45.0%) by honey bees and 232 (2.7%) flower visits by leafcutting bees. The average seed set for the 153 plants in this study was (mean ± STD) 754.67 ± 986.90, with a range from 6.63 to 9,874.62 seeds per plant. The plants in the experiment had (mean ± STD) 4.93 ± 3.41 racemes per stem with a range between 0.11 and 14.6 racemes per plant. Each value for a plant represents the average of ten readings per plant. Plants had 7.53 ± 2.44 open flowers per raceme with a range from 3 to 14.4 open flowers per raceme and 30.65 ± 16.4 stems per plant with a range between 6 and 87 stems. Flower color varied with (mean ± STD) chroma values of 1.29 ± 0.76 with a range from 0.257 to 3.48; reflectivity values of 3.76 ± 1.11 and a range from 1.76 to 7.14 and hue values of -0.012 ± 0.476 and a range from -0.802 to 1.21.

[1]https://github.com/yoavzemel/dsd

The opportunity for selection was 1.74 for overall fitness, calculated using total seed set per plant. When RF was based on the proportion of flower visits, the opportunity for selection was 1.24 for bumble bee, 2.53 for honey bee and 46.48 for leafcutting bee. The average seed set attributable to each bee species, based on proportion of flower visits, was 396.27 seeds per plant for bumble bee; 261.03 for honey bee; and 61.68 for leafcutting bee. When RF was based on the proportion of tripped flowers, the opportunity for selection was 1.15 for bumble bee, 3.16 for honey bee and 35.54 for leafcutting bee. The average seed set of a plant attributable to each bee species was 453.32 seeds for bumble bee, 187.24 seeds for honey bee, and 84.25 seeds for leafcutting bee.

Phenotypic Selection

All Bees Combined

Over all pollinators combined, we observed a positive directional selection differential *S* and selection gradient β on the number of stems per plant indicating selection to increase the number of stems per plant (**Table 2**). For the number of racemes per stem, there was a statistically significant positive directional selection differential *S* and selection gradient β indicative of selection for an increase in the number of racemes. However, we also detected a statistically significant negative quadratic selection differential C_{22} although the quadratic selection gradient γ_{ii} was not statistically significant, suggesting indirect non-linear selection on the number of racemes per stem (**Table 2**). Finally, there was a statistically significant positive correlational selection between the number of open flowers per raceme and the chroma or darkness of flowers (γ_{FlrChr}), at least for the regression model with log transformed RF (**Table 2** and **Figure 1A**). Under correlational selection, particular combinations of two traits expressed together in the same individual are favored and here pollinators favored plants with more open flowers per raceme and darker flowers.

Bumble Bee

For bumble bees, the positive directional selection differential *S* and gradient β were both statistically significant for the number of racemes per stem and for the number of stems per plant suggesting selection to increase both traits (**Table 3**). In addition, we observed a statistically significant positive correlational selection gradient between the number of open flowers on a raceme and the darkness of a flower (chroma) (γ_{FlrChr}) for all cases except when RF was based on the number of tripped flowers and the statistical significance of selection coefficients were tested using bootstrapping (**Table 3**). Bumble bees favored plants with more open flowers per raceme and with darker flowers (**Figure 1B**). There was also a positive correlational selection gradient between the number of open flowers and flower reflectivity (γ_{FlrRef}) but it was only statistically significant when RF was based on the proportion of flowers visited and when log transformed RF regression model was used to detect the significance of the selection coefficients (**Table 3**).

Honey Bee

For honey bee, there was a statistically significant negative quadratic selection differential (C_{22}) and quadratic selection

TABLE 3 | Phenotypic selection for bumble bee with relative fitness calculated based either on the proportion of flowers visited or of flowers tripped by bumble bees.

N = 153		Proportion of flowers visited					Proportion of tripped flowers				
		Log transformed		Bootstrap			Log transformed		Bootstrap		
Trait	Selection coefficient	Estimate	*P*	Estimate	Lower 95%	Upper 95%	Estimate	*P*	Estimate	Lower 95%	Upper 95%
Racemes per Stem	S	0.276	<0.0001	0.276	0.137	0.423	0.283	<0.0001	0.283	0.157	0.420
Racemes per Stem	β	0.223	0.006	0.223	0.053	0.405	0.212	<0.0001	0.212	0.070	0.371
Stems per plant	S	0.434	<0.0001	0.434	0.190	0.733	0.475	<0.0001	0.475	0.226	0.769
Stems per plant	β	0.396	<0.0001	0.396	0.146	0.703	0.437	<0.0001	0.437	0.183	0.731
Open Flowers per raceme	γ_{FlrChr}	0.706	0.011	0.706	0.008	1.24	0.604	0.018	0.604	NS	NS
Open Flowers per raceme	γ_{FlrRef}	0.588	0.032	0.588	NS	NS	0.115	NS	0.115	NS	NS

The statistical significance of the selection coefficients was determined using either regression models with log transformed relative fitness or using bootstrapping. The methodology followed to obtain the different selection coefficients is summarized in ***Table 1****. The letter P stands for probability and NS for not statistically significant. Flr stands for open flowers per raceme, Chr for flower chroma and Ref for flower reflectivity.*

TABLE 4 | Phenotypic selection for honey bee with relative fitness calculated using either the proportion of flowers visited or the proportion of flowers tripped by honey bees.

N = 153		Proportion of flowers visited					Proportion of flowers tripped				
		Log transformed		Bootstrap			Log transformed		Bootstrap		
Trait	Selection coefficient	Estimate	*P*	Estimate	Lower 95%	Upper 95%	Estimate	*P*	Estimate	Lower 95%	Upper 95%
Racemes per Stem	S	0.314	0.004	0.314	0.108	0.548	0.292	0.011	0.292	0.084	0.579
Racemes per Stem	C_{22}	−0.268	<0.0001	−0.268	−0.443	−0.157	−0.256	0.002	−0.256	−0.455	−0.132
Racemes per Stem	$\gamma_{rcpsrcps}$	−0.234	0.01	−0.234	−0.450	−0.057	−0.223	0.026	−0.223	−0.465	−0.009
Stems per plant	S	0.907	<0.0001	0.907	0.605	1.262	0.951	<0.0001	0.951	0.649	1.379
Stems per plant	β	0.890	<0.0001	0.890	0.580	1.273	0.947	<0.0001	0.745	0.489	1.174
Stems per plant	C_{22}	0.186	NS	0.186	0.012	0.400	0.196	NS	0.196	0.017	0.349
Stems per plant	$\gamma_{stpstpp}$	0.230	NS	0.230	0.025	0.536	0.246	NS	0.246	0.028	0.598

The statistical significance of the selection coefficients was determined using either regression models with log transformed relative fitness or using bootstrapping. The methodology used to obtain the different selection coefficients is summarized in ***Table 1****. The letter P stands for probability and NS for not statistically significant. Rcps stands for racemes per stem and stpp stands for stems per plant.*

gradient (γ_{ii}) for the number of racemes per stem indicative of non-linear selection (**Table 4**). Results of the spline regression analysis indicates that honey bees exert some stabilizing selection on the number of racemes per stem (**Figure 2A**). For the number of stems per plant, both the positive directional selection differential *S* and selection gradients β were statistically significant but we also detected non-linear positive selection, suggestive of disruptive selection, with statistically significant quadratic selection differential (C_{22}) and gradient (γ_{ii}) but only when bootstrapping was used to determine the statistical significance of the selection coefficients (**Table 4**). The coefficient of directional selection was much larger than the non-linear selection coefficient (**Table 4**) which translated into mostly directional selection for increased number of stems per plant as indicated by the spline regression analysis (**Figure 2B**). Patterns were similar whether RF was based on the proportion of visited or tripped flowers (**Table 4**).

Leafcutting Bee

For leafcutting bee, only bootstrapping was used to determine the statistical significance of the selection coefficients. For the number of racemes per stem, we detected both directional and non-linear selection (**Table 5**). There was a statistically significant positive selection differential *S* and gradient β but also a statistically significant negative quadratic selection differential C_{22} and gradient γ_{ii} at least when RF was based on the proportion of visited flowers (**Table 5**). The spline analysis indicated that leafcutting bees exerted some stabilizing selection on the number of racemes per plant (**Figure 3A**). For the number of stems per plant, we detected a positive directional selection differential *S* and gradient β favoring plants with more stems. Finally, we observed a statistically significant negative quadratic selection gradient γ_{ii} for flower color or hue, indicating some stabilizing selection on hue by leafcutting bees (**Table 5** and **Figure 3B**).

Distributional Selection Differential

When performing distributional selection differential (DSD) analyses, we detected positive directional selection for the number of racemes per stem and the number of stems per plant for all bees combined and for each bee species (**Table 6**). We did not detect non-linear selection on any components of floral display size and did not detect selection on any components of flower color for either all bees combined or any of the bee species (**Table 6**). We present the DSD results to contrast with the results obtained using the Lande and Arnold (1983) approach. We will leave other studies to discuss discrepancies between approaches and below concentrate on the results obtained using the more traditional method originally proposed by Lande and Arnold (1983).

DISCUSSION

Selection on Flower Color Relative to Floral Display Size

The number of stems per plant and the number of racemes per stem were selected by all three bee species. In contrast,

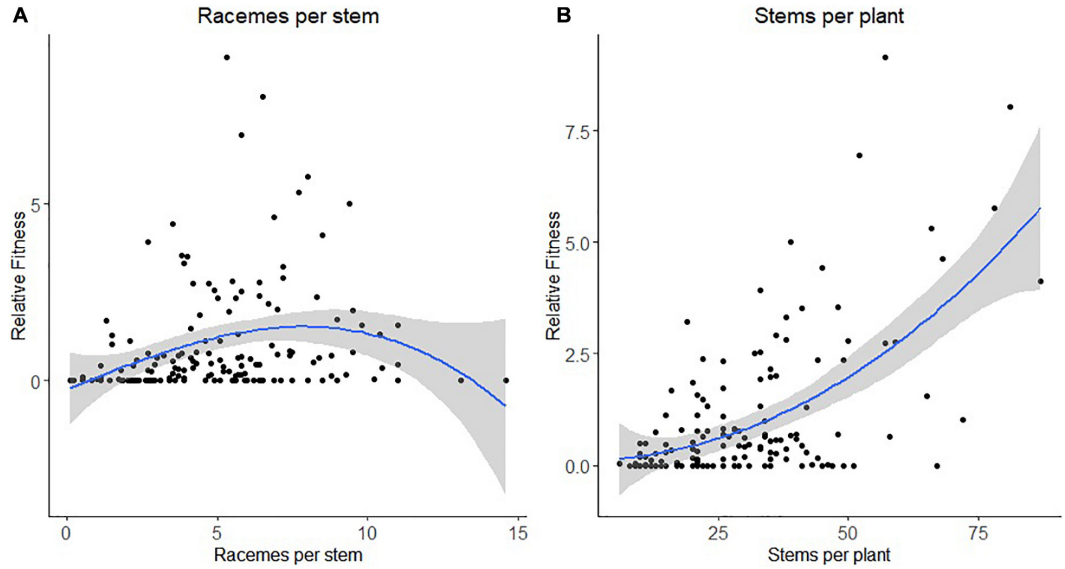


FIGURE 2 | Non-linear selection by honey bee for panel **(A)** the number of racemes per stem and **(B)** the number of stems per plant. The blue line represents the predictive spline curve while the gray area encompasses the 95% confidence intervals.

only leafcutting bees favored intermediate hue and bumble bees exhibited correlational selection between open flowers per raceme and flower chroma. Components of floral display size, plant-level attractants, were selected more consistently relative to components of flower color, a flower-level attractant (sensu Caruso et al., 2019). This trend was observed under conditions favorable to detect phenotypic selection (Sahli and Conner, 2011). The plants used in this study exhibited a high level of phenotypic variation in both flower color and floral display size and the variation in flower color was greater than typically occurs in wild *M. sativa* populations. We also observed strong opportunity for selection overall and within each pollinator species.

The foraging behavior of pollinators may help explain the difference between selection on plant-level and flower-level attractants. Pollinators forage for rewards and their goal is to collect pollen and nectar to provide for their young and feed themselves. Components of floral display size such as the number of racemes per stem and the number of stems per plant are both indicative of the amount of resources available on a plant. Bumble bees can determine whether a flower offers pollen or not and can detect the number of pollen-producing flowers on a plant. They are attracted to inflorescences, a plant-level attractant, based on the number of pollen-producing flowers (Brunet et al., 2015). Similarly, bumble bees may be able to detect the number of nectar-producing flowers on inflorescences (Makino and Sakai, 2007). Bumble bees, on the other hand, cannot distinguish between flowers presenting distinct amount of pollen, a flower-level attractant, unless it is linked to another trait such as flower size or flower color (Brunet et al., 2015; Thairu and Brunet, 2015). Similarly, while bees have innate preferences for flower color (Simonds and Plowright, 2004; Raine and Chittka, 2007), they learn to associate a flower color with a reward and can switch their preference of flower color for the color providing the most reward (Ings et al., 2009; Thairu and Brunet, 2015). The fact that plant-level attractants such as floral display size directly advertise resource availability to pollinators may help explain why, relative to flower-level attractants, they are more likely to be selected by pollinators within plant populations.

An association between a reward and flower color is more likely to occur among plant populations or plant species of distinct colors rather than within a population where the association between a color and a reward can be broken down by recombination (Brunet et al., 2015). This may help explain why flower color polymorphisms are more common among than within plant populations (Narbona et al., 2018). Pollinators have been suggested as the selective agents responsible for flower color polymorphisms among populations (Streisfeld and Kohn, 2007) and in some cases the genes responsible for the change in flower color associated with each pollinator have been elucidated (Streisfeld et al., 2013). Similarly, the genetic basis of flower color differences has been elucidated for some plant species and shown to be responsible for the pollinator preference (Bradshaw and Schemske, 2003; Hoballah et al., 2007). However, it remains unclear whether the pollinator preference created the flower color diversification or whether the association between flower color and pollinator arose following the fixation of the flower color in the population or species due to a different factor.

Within plant populations, correlational selection between flower color and floral display size may facilitate the evolution of flower color via pollinators. Correlational selection, as was observed in the current study between number of open flowers per raceme and flower chroma, provides a mechanism to associate a flower-level attractant like flower color to a plant-level attractant that advertise resource availability to a pollinator. Moreover, correlational selection leads to the development of genetic correlations between traits (Roff and Fairbairn, 2012) and

TABLE 5 | Phenotypic selection for leafcutting bee with relative fitness calculated using either the proportion of flowers visited or the proportion of flowers tripped by leafcutting bees.

N = 153		Proportion of flowers visited			Proportion of flowers tripped		
Trait	**Selection coefficient**	**Estimate**	**Lower 95%**	**Upper 95%**	**Estimate**	**Lower 95%**	**Upper 95%**
Racemes per Stem	S	0.512	0.167	1.231	0.501	0.159	1.090
Racemes per Stem	β	0.426	0.117	1.433	0.387	0.108	1.024
Racemes per Stem	C_{22}	−0.235	−0.631	−0.042	−0.226	−0.532	−0.033
Racemes per Stem	$\gamma_{rcpsrcps}$	−0.249	−1.007	−0.001	−0.249	NS	NS
Stems per plant	S	0.724	0.314	1.585	0.791	0.338	1.950
Stems per plant	β	0.628	0.216	1.435	0.696	0.244	1.703
Hue	γ_{huehue}	−0.620	−1.940	−0.068	−0.628	−1.810	−0.063

The statistical significance of the selection coefficients was only determined using bootstrapping due to the high number of plants with no leafcutting bee visits. The methodology used to obtain the different selection coefficients is summarized in ***Table 1****. The abbreviation NS stands for not statistically significant. Rcps stands for racemes per stem and hue for flower hue.*

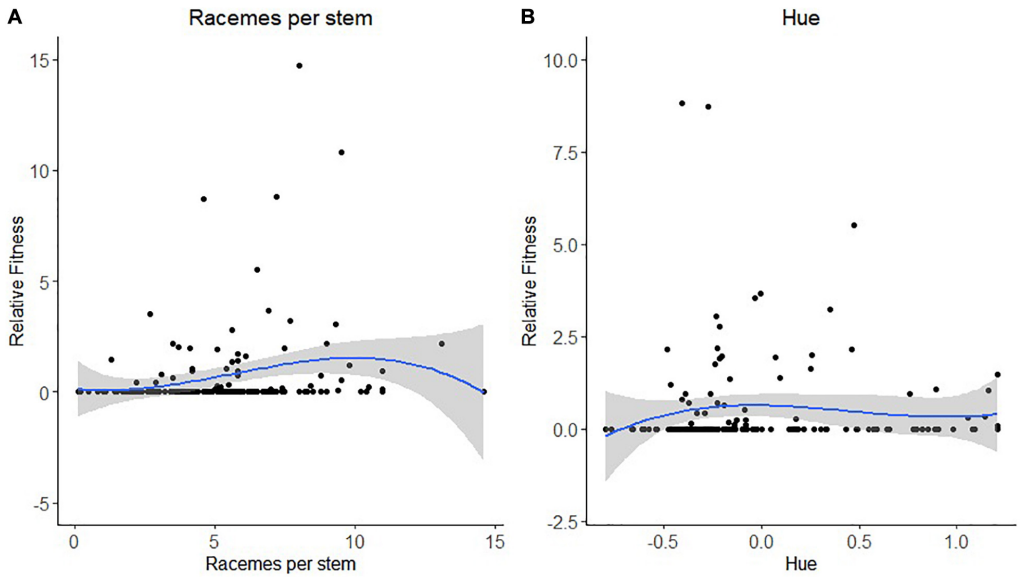


FIGURE 3 | Non-linear selection by leafcutting bees for **(A)** the number of racemes per stem and **(B)** flower hue or color. The blue line represents the predictive spline curve while the gray area encompasses the 95% confidence intervals.

correlational selection between a flower color and floral display size has been shown to increase the frequency of a color morph within a population even in the absence of differences between color morphs in seedling germination or survival (Gomez, 2000). Correlational selection by pollinators, between flower color and a plant-level attractant, may facilitate the maintenance of flower color polymorphism within plant populations. The role or correlational selection in the evolution of flower color in plant populations deserves more attention.

Distinct Pollinators and Selection of Floral Traits

Distinct pollinators can exert different or conflicting selection on floral traits (Galen, 1989; Sahli and Conner, 2011; Kulbaba and Worley, 2013) and in the current study, we found different patterns of selection on some floral traits by the distinct bee species. For example, bumble bees exerted positive directional selection on the number of racemes per stem and favored plants with more racemes per stem while both honey bees and leafcutting bees exerted non-linear selection in the form of stabilizing selection favoring an intermediate number of racemes per stem. Distinct pollinators may also select on distinct floral traits. The common eastern bumble bee was the only bee species favoring darker flowers, when associated with racemes with more open flowers. Bumble bees were associated with correlational selection between these two floral traits. Leafcutting bees favored intermediate flower hue and honey bees did not select on any components of flower color. Finally, distinct pollinators can select on some floral traits in a similar way as we observed here with all three bee species favoring plants with more stems.

While illustrating how distinct pollinators can differentially or similarly influence floral traits, this study also links the selection by the three pollinators to the overall selection on the floral

TABLE 6 | Distributional selection differential on the floral traits.

Floral Traits	DSD	Probability	dD	Probability	dN	Probability	S	Probability	delta δ	Beta β
All bees										
Racemes per stem	**0.333**	**0.002**	**0.328**	**0.002**	0.006	0.878	**0.328**	**0.002**	0.278	0.193
Stems per plant	**0.806**	**0.000**	**0.806**	**0.000**	0.000	0.984	**0.806**	**0.000**	0.732	0.786
Total flowers	0.060	0.973	0.017	0.863	0.043	0.398	0.017	0.863	0.615	−0.031
Hue	0.064	0.911	0.006	0.955	0.058	0.173	0.006	0.955	0.492	0.016
Chroma	0.050	0.990	0.010	0.931	0.040	0.353	−0.010	0.931	0.241	−0.115
Reflectivity	0.088	0.774	0.040	0.709	0.047	0.345	0.040	0.709	0.214	0.038
Bumble bee										
Racemes per stem	**0.276**	**0.002**	**0.276**	**0.002**	0.000	0.983	**0.276**	**0.002**	0.178	0.223
Stems per plant	**0.439**	**0.000**	**0.434**	**0.000**	0.005	0.871	**0.434**	**0.000**	0.399	0.396
Total flowers	0.064	0.904	0.057	0.538	0.008	0.825	0.057	0.538	0.120	0.008
Hue	0.061	0.881	0.010	0.915	0.051	0.194	0.010	0.915	1.463	0.120
Chroma	0.065	0.842	0.038	0.679	0.027	0.474	0.038	0.679	−0.063	−0.119
Reflectivity	0.116	0.347	0.086	0.346	0.030	0.504	−0.086	0.346	0.149	−0.184
Honey bee										
Racemes per stem	**0.370**	**0.004**	**0.314**	**0.016**	0.055	0.429	**0.314**	**0.016**	0.229	0.132
Stems per plant	**0.907**	**0.000**	**0.907**	**0.000**	0.000	0.983	**0.907**	**0.000**	0.849	0.890
Total flower	0.156	0.408	0.142	0.275	0.014	0.790	0.142	0.275	0.111	0.065
Hue	0.159	0.339	0.118	0.367	0.041	0.452	0.118	0.367	0.239	0.140
Chroma	0.116	0.642	0.111	0.395	0.006	0.857	0.111	0.395	0.124	0.091
Reflectivity	0.093	0.901	0.061	0.634	0.032	0.618	0.061	0.634	0.336	0.122
Leafcutting bee										
Racemes per stem	**0.543**	**0.079**	**0.512**	**0.076**	0.031	0.800	**0.512**	**0.076**	0.507	0.426
Stems per plant	**0.734**	**0.016**	**0.724**	**0.015**	0.010	0.894	**0.724**	**0.015**	0.557	0.628
Total flowers	0.223	0.853	0.157	0.586	0.067	0.611	−0.157	0.586	0.313	−0.262
Hue	0.155	0.979	0.037	0.904	0.117	0.328	−0.037	0.904	2.529	0.204
Chroma	0.323	0.414	0.275	0.351	0.048	0.640	0.275	0.351	0.565	0.149
Reflectivity	0.179	0.967	0.131	0.656	0.048	0.703	−0.131	0.656	−0.181	−0.042

Relative fitness was based on the proportion of floral visits. Total selection is measured by DSD while dD represents the directional and dN the non-directional component of selection. The general selection differential is S and β is the selection gradient. Statistically significant values are bolded.

traits. Overall, there is directional selection on the number of racemes per stem and stems per plant with indirect non-linear selection on racemes per stem. There is also correlational selection between number of open flowers per raceme and flower chroma. Clearly bumble bees are solely responsible for the correlational selection while all three bee species exert directional selection on the number of stems per plant. While both honey bees and leafcutting bees exert some stabilizing selection on the number of racemes per stem, the overall selection is mostly directional. Bumble bees were the most abundant pollinators and better trippers than honey bees, the second most frequent visitors. The differential influence of the three bee species on floral traits indicates that the overall pattern of selection in a population will vary with the abundance and efficiency of its pollinators. We therefore, expect temporal or spatial variation in pollinators (Brunet, 2009; Narbona et al., 2018) to influence the temporal or spatial pattern of selection on floral traits (Kelly, 1992; Siepielski et al., 2009, 2013; Narbona et al., 2018). However, environmental factors may also vary among populations or temporally within populations and affect floral trait evolution (Schemske and Bierzychudek, 2001; Strauss and Whittall, 2006; Caruso et al., 2019). Interestingly, yearly variation in abiotic factors can modify the pattern of correlational selection (Maad, 2000). Both pollinators and abiotic factors should be considered when examining phenotypic selection of floral traits over time or space (Narbona et al., 2018; Sletvold, 2019).

RF and Phenotypic Selection Within Bee Species

The methodology introduced in this study permits evaluation of phenotypic selection by distinct pollinators simultaneously, using the same set of plants. It more realistically describes the process of pollinator-mediated selection in natural populations. Sample sizes remain the same, over all bees and within each bee species, although the proportion of flowers not visited by a bee species may vary among bee species. Of interest is the fact that pattern of selection obtained over combined pollinators could be explained by the patterns observed for each bee species. Moreover, some selection patterns were only significant at the level of a bee species. For example, selection on flower color by leafcutting bees was not expressed at the whole plant level, likely because leafcutting bees were not as common in the study and were responsible for a lesser proportion of the seeds produced by the plants in the population.

We assigned the selection patterns observed in this study to pollinators rather than to another biotic or to abiotic factors. This approach was followed because the number of pollinator visits increase seed set in this plant species (Bauer et al., 2017); *M. sativa* plants set few seeds in the absence of pollinators (Bohart, 1957); plants were grown in a common environment minimizing variation in resource availability; and herbivory was not observed. Gathering pollination data in phenotypic selection studies will provide useful information on pollinator-mediated selection by distinct pollinators. We will further argue in a separate manuscript that comparing selection gradients between hand-pollinated and open-pollinated plants may not be the most efficient method to assign selection to pollinators (Brunet, in preparation).

The approach introduced in this study relies on good quality pollinator data and a link between visitation and seed set. The pollinator visitation data should be representative of the plant species under study over its flowering season. The plants used to collect pollinator data should represent the variation in floral display size that occurs spatially and temporally in the population. If pollinator types vary throughout the day or the flowering season, one should sample to reflect such variation. To link floral visits to seed set, it is best to sample seeds on visited plants after a period that reflects the time it takes for seeds to reach maturity. Finally, while applied to female reproductive success, the methodology could be extended to male reproductive success. In this case the proportion of floral visits to plant(i) is used for proportional visits by the distinct pollinators as it reflects the pollen leaving plant(i). The total seeds assignable to plant(i), on plant (i) if selfing occurs and on other plants in the population, represent the seed set for male function for plant(i). Results of this study illustrate how the approach proposed can attribute overall phenotypic selection patterns to individual pollinators and we therefore advocate the approach introduced here for future studies examining the impact of distinct pollinators on floral trait evolution.

CONCLUSION

The methodology introduced to isolate and combine the phenotypic selection patterns of distinct bee species on floral traits provides patterns of selection similar to what has been observed in previous studies. The selection patterns observed over all bees could be assigned to specific bee species. All three bee species selected for components of floral display sizes but not all bees favored components of flower color although the selection coefficients were strong. This difference between plant-level and flower-level attractants could be explained by the fact that floral display size but not flower color directly advertises resource availability to pollinators. Spatial and temporal variation in the abundance of the distinct pollinators is expected to affect patterns of selection of flower traits, particularly for traits differentially selected by the distinct pollinators. Correlational selection between floral display size, a plant-level attractant, and flower color, a flower-level attractant, is expected to facilitate the evolution of flower color by pollinators within plant populations. Studies of pollinator-mediated selection would benefit from combining data on pollinator visitation rates together with seed set and measurements of floral traits when examining the impact of distinct pollinators on floral trait evolution.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JB conceived the study and wrote the manuscript. AB collected the data and samples from the field, processed the samples, and did preliminary data analyses. AF performed the data analyses for the manuscript, prepared the Figures, and provided comments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Arnold, S. J., and Wade, M. J. (1984). On the measurement of natural and sexual selection: theory. *Evolution (N. Y).* 38, 709–719. doi: 10.1111/j.1558-5646.1984.tb00344.x

Auguie, B. (2017). *gridExtra: Miscellaneous Functions for "Grid" Graphics. R Packag. Version 2.3*. Available online at: https://cran.r-project.org/package=gridExtra (accessed May 22, 2020).

Bauer, A. A., Clayton, M. K., and Brunet, J. (2017). Floral traits influencing plant attractiveness to three bee species: consequences for plant reproductive success. *Am. J. Bot.* 104, 772–781. doi: 10.3732/ajb.1600405

Bohart, G. E. (1957). Pollination of alfalfa and red clover. *Annu. Rev. Entomol.* 2, 355–380. doi: 10.1146/annurev.en.02.010157.002035

Bradshaw, H. D., and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkey flowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Brodie, E. D. (1992). Correlational selection for color pattern and antipredator behavior in the garter snake *Thamnophis ordinoides*. *Evolution (N. Y).* 46, 1284–1298. doi: 10.1111/j.1558-5646.1992.tb01124.x

Brodie, E. D., and Janzen, F. J. (1996). On the assignment of fitness values in statistical analyses of selection. *Evolution (N. Y).* 50, 437–442. doi: 10.1111/j.1558-5646.1996.tb04505.x

Brookes, B., Small, E., Lefkovitch, L. P., Damman, H., and Fairey, D. T. (1994). Attractiveness of alfalfa (*Medicago sativa* L.) to wild pollinators in relation to wildflowers. *Can. J. Plant Sci.* 74, 779–783. doi: 10.4141/cjps94-139

Brunet, J. (2009). Pollinators of the rocky mountain columbine: temporal variation, functional groups and associations with floral traits. *Ann. Bot.* 103, 1567–1578. doi: 10.1093/aob/mcp096

Brunet, J., and Stewart, C. M. (2010). Impact of bee species and plant density on alfalfa pollination and potential for gene flow. *Psyche (Lond.)* 2010:201858. doi: 10.1155/2010/201858

Brunet, J., Thairu, M. W., Henss, J. M., Link, R. I., and Kluever, J. A. (2015). The effects of flower, floral display, and reward sizes on bumblebee foraging behavior when pollen is the reward and plants are dichogamous. *Int. J. Plant Sci.* 176, 811–819. doi: 10.1086/683339

Brunet, J., Zhao, Y., and Clayton, M. K. (2019). Linking the foraging behavior of three bee species to pollen dispersal and gene flow. *PLoS One* 14:e0212561. doi: 10.1371/journal.pone.0212561

Campbell, D. R., Waser, N. M., and Meléndez-Ackerman, E. J. (1997). Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *Am. Nat.* 149, 295–315. doi: 10.1086/285991

Cane, J. H. (2009). Pollinating bees (Hymenoptera: Apiformes) of U.S. Alfalfa compared for rates of pod and seed set. *J. Econ. Entomol.* 95, 22–27. doi: 10.1603/0022-0493-95.1.22

Canty, A., and Ripley, B. (2020). *boot: Bootstrap R (S-Plus) Functions*. R Package Version 1.3-25.

Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution (N. Y).* 73, 4–14. doi: 10.1111/evo.13639

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ON: Enviroquest Ltd.), 157–196.

Dart, S.R., Samis, K. E. A., Austen, E., and Eckert, C. G. (2012). Broad geographic covariation between floral traits and the mating system in Camissoniopsis cheiranthifolia (Onagraceae): multiple stable mixed mating systems across the species range? Ann. Bot. 109, 599–611. doi: 10.1093/aob/mcr266

Davison, A., and Hinkley, D. (1997). *Bootstrap Methods and their Application*. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511802843

Endler, J. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biol. J. Linn. Soc.* 41, 315–352. doi: 10.1111/j.1095-8312.1990.tb00839.x

Fishman, L., and Willis, J. H. (2008). Pollen limitation and natural selection on floral characters in the yellow monkeyflower, Mimulus guttatus. *New Phytol.* 177, 802–810. doi: 10.1111/j.1469-8137.2007.02265.x

Galen, C. (1989). Measuring pollinator-mediated selection on morphometric floral traits: bumblebees and the alpine sky pilot, *Polemonium viscosum*. *Evolution (N. Y).* 43, 882. doi: 10.2307/2409315

Galen, C. (1996). Floral evolution: adaptation to bumblebee pollination in an alpine wildflower, *Polemonium viscosum*. *Evolution (N. Y).* 50, 120–125. doi: 10.2307/2411183

Gomez, J. M. (2000). Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *J. Evol. Biol.* 13, 689–699. doi: 10.1046/j.1420-9101.2000.00196.x

Harder, L. D., and Johnson, S. D. (2009). Tansley review Darwin ' s beautiful contrivances: evolutionary and functional evidence. *New Phytol.* 183, 530–545. doi: 10.1111/j.1469-8137.2009.02914.x

Henshaw, J. M., and Zemel, Y. (2017). A unified measure of linear and nonlinear selection on quantitative traits. *Methods Ecol. Evol.* 8, 604–614. doi: 10.1111/2041-210X.12685

Hoballah, M. E., Gübitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., et al. (2007). Single gene-mediated shift in pollinator attraction in *Petunia*. *Plant Cell* 19, 779–790. doi: 10.1105/tpc.106.048694

Ings, T. C., Raine, N. E., and Chittka, L. (2009). A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 63, 1207–1218. doi: 10.1007/s00265-009-0731-8

Kelly, C. (1992). Spatial and temporal variation in selection on correlated life-history traits and plant size in *Chamaecrista fasciculata*. *Evolution (N. Y).* 46, 1658–1673. doi: 10.2307/2410022

Kulbaba, M. W., and Worley, A. C. (2012). Selection on floral design in polemonium brandegeei (Polemoniaceae): female and male fitness under hawkmoth pollination. *Evolution (N. Y).* 66, 1344–1359. doi: 10.1111/j.1558-5646.2011.01536.x

Kulbaba, M. W., and Worley, A. C. (2013). Selection on *Polemonium brandegeei* (Polemoniaceae) flowers under hummingbird pollination: in opposition, parallel or independent of selection by hawkmoths? *Evolution (N. Y).* 67, 2194–2206. doi: 10.1111/evo.12102

Lande, R., and Arnold, S. J. (1983). The Measurement of selection on correlated characters. *Evolution (N. Y).* 37, 1210. doi: 10.2307/2408842

Maad, J. (2000). Phenotypic selection in Hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution (N. Y).* 54, 112–123. doi: 10.1554/0014-3820(2000)054[0112:psihpp]2.0.co;2

Makino, T. T., and Sakai, S. (2007). Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. *Funct. Ecol.* 21, 854–863. doi: 10.1111/j.1365-2435.2007.01293.x

Mitchell-Olds, T., and Shaw, R. G. (1987). Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution (N. Y).* 41, 1149–1161. doi: 10.1111/j.1558-5646.1987.tb02457.x

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Parachnowitsch, A. L., and Kessler, A. (2010). Pollinators exert natural selection on flower size and floral display in *Penstemon digitalis*. *New Phytol.* 188, 393–402. doi: 10.1111/j.1469-8137.2010.03410.x

Pitts-Singer, T. L., and Cane, J. H. (2011). The alfalfa leafcutting bee, megachile rotundata: the world's most intensively managed solitary bee. *Annu. Rev. Entomol.* 56, 221–237. doi: 10.1146/annurev-ento-120709-144836

R Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raine, N. E., and Chittka, L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee Bombus terrestris. *PLoS One* 2:556. doi: 10.1371/journal.pone.0000556

Roff, D. A., and Fairbairn, D. J. (2012). A test of the hypothesis that correlational selection generates genetic correlations. *Evolution (N. Y).* 66, 2953–2960. doi: 10.1111/j.1558-5646.2012.01656.x

Sahli, H. F., and Conner, J. K. (2011). Testing for conflicting and nonadditive selection: floral adaptation to multiple pollinators through male and female fitness. *Evolution (N. Y).* 65, 1457–1473. doi: 10.1111/j.1558-5646.2011.01229.x

Sandring, S., and Ågren, J. (2009). Pollinator-mediated selection on floral display and flowering time in the perennial herb arabidopsis lyrata. *Evolution (N. Y).* 63, 1292–1300. doi: 10.1111/j.1558-5646.2009.00624.x

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution (N. Y).* 55, 1269–1282. doi: 10.1111/j.0014-3820.2001.tb00650.x

Siepielski, A. M., Dibattista, J. D., and Carlson, S. M. (2009). It's about time: the temporal dynamics of phenotypic selection in the wild. *Ecol. Lett.* 12, 1261–1276. doi: 10.1111/j.1461-0248.2009.01381.x

Siepielski, A. M., Gotanda, K. M., Morrissey, M. B., Diamond, S. E., DiBattista, J. D., and Carlson, S. M. (2013). The spatial patterns of directional phenotypic selection. *Ecol. Lett.* 16, 1382–1392. doi: 10.1111/ele.12174

Simonds, V., and Plowright, C. (2004). How do bumblebees first find flowers? Unlearned approach responses and habituation. *Anim. Behav.* 67, 379–386. doi: 10.1016/j.anbehav.2003.03.020

Sletvold, N. (2019). The context dependence of pollinator-mediated selection in natural populations. *Int. J. Plant Sci.* 180, 934–943. doi: 10.1086/705584

Sletvold, N., Tye, M., and Ågren, J. (2017). Resource- and pollinator-mediated selection on floral traits. *Funct. Ecol.* 31, 135–141. doi: 10.1111/1365-2435.12757

Smith, S. D. (2014). Quantifying color variation: improved formulas for calculating hue with segment classification. *Appl. Plant Sci.* 2:1300088. doi: 10.3732/apps.1300088

Stinchcombe, J. R., Agrawal, A. F., Hohenlohe, P. A., Arnold, S. J., and Blows, M. W. (2008). Estimating nonlinear selection gradients using quadratic regression coefficients: double or nothing? *Evolution (N. Y).* 62, 2435–2440. doi: 10.1111/j.1558-5646.2008.00449.x

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. Barrett (Oxford: Oxford University Press), 120–132.

Streisfeld, M. A., and Kohn, J. R. (2007). Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evol. Biol.* 20, 122–132. doi: 10.1111/j.1420-9101.2006.01216.x

Streisfeld, M. A., Young, W. N., and Sobel, J. M. (2013). Divergent Selection drives genetic differentiation in an R2R3-MYB transcription factor that contributes to incipient speciation in *Mimulus aurantiacus*. *PLoS Genet.* 9:e1003385. doi: 10.1371/journal.pgen.1003385

Thairu, M. W., and Brunet, J. (2015). The role of pollinators in maintaining variation in flower colour in the rocky mountain columbine, *Aquilegia coerulea*. *Ann. Bot.* 115, 971–979. doi: 10.1093/aob/mcv028

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis. Secondi*. New York, NY: Springer Verlag. doi: 10.1007/978-3-319-24277-4

Wood, S. N. (2011). Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *J. R. Stat. Soc. Ser. B Stat. Methodol.* 73, 3–36. doi: 10.1111/j.1467-9868.2010.00749.x

Zhao, Z. G., and Wang, Y. K. (2015). Selection by pollinators on floral traits in generalized *Trollius ranunculoides* (Ranunculaceae) along altitudinal gradients. *PLoS One* 10:e0118299. doi: 10.1371/journal.pone.0118299

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frontiers in Plant Science

REVIEW
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Fragmentary Blue: Resolving the Rarity Paradox in Flower Colors

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Blue is a favored color of many humans. While blue skies and oceans are a common visual experience, this color is less frequently observed in flowers. We first review how blue has been important in human culture, and thus how our perception of blue has likely influenced the way of scientifically evaluating signals produced in nature, including approaches as disparate as Goethe's Farbenlehre, Linneaus' plant taxonomy, and current studies of plant-pollinator networks. We discuss the fact that most animals, however, have different vision to humans; for example, bee pollinators have trichromatic vision based on UV-, Blue-, and Green-sensitive photoreceptors with innate preferences for predominantly short-wavelength reflecting colors, including what we perceive as blue. The subsequent evolution of blue flowers may be driven by increased competition for pollinators, both because of a harsher environment (as at high altitude) or from high diversity and density of flowering plants (as in nutrient-rich meadows). The adaptive value of blue flowers should also be reinforced by nutrient richness or other factors, abiotic and biotic, that may reduce extra costs of blue-pigments synthesis. We thus provide new perspectives emphasizing that, while humans view blue as a less frequently evolved color in nature, to understand signaling, it is essential to employ models of biologically relevant observers. By doing so, we conclude that short wavelength reflecting blue flowers are indeed frequent in nature when considering the color vision and preferences of bees.

Keywords: blue, flower color, biogeography, elevation, land use, plant diversity, productivity

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INTRODUCTION

Why make so much of fragmentary blue
In here and there a bird, or butterfly,
Or flower, or wearing-stone, or open eye,
When heaven presents in sheets the solid hue?

Since earth is earth, perhaps, not heaven (as yet)–
Though some savants make earth include the sky;
And blue so far above us comes so high,
It only gives our wish for blue a whet. (Frost, 1920)

When we as human observers use our color vision to document the natural world, we need to be cognizant of the limitations and biases of our perception. In this synthesis review, we consider the reported relative rarity of blue flowers in many ecological studies, and subsequently discuss how a different view of flower spectral data can be obtained by considering the vision of major pollinators with the goal of bridging different fields to navigate toward the frontiers of plant color science.

Studies in psychophysics on adult humans from many countries show that blue is the most frequently preferred color hue (Granger, 1952; McManus et al., 1981; Ou et al., 2004). A blue color preference is also observed in human infant studies (Teller et al., 2004; Zemach et al., 2007), although infant color vision experiments show evidence for a preference to reddish hues in some contexts (Franklin et al., 2010). An ecological explanation for our blue preference is that we like clear sky and blue water and increasingly develop a preference for those from young childhood (Palmer and Schloss, 2010), and indeed color preferences in humans are frequently influenced by important environmental factors in our lives (Palmer et al., 2013).

The color blue has long been highly valued throughout the history of humans. In ancient Egypt, the combination of silica (SiO_2), calcium oxide (CaO), and copper oxide (CuO) was used to make Egyptian blue (*irtyu*), a long-lasting entrancing pigment representing the color of the sky and heavens that was used for the decoration of statues that can still be observed (Eastaugh et al., 2004). The earliest known use of blue dyes can be traced to ancient Peru where an indigoid dye (indigotin E132), was used to dye cotton fabric about 6000 years ago, about 1500 years before the first evidences of usage of blue fabric dyes in ancient Egypt (Splitstoser et al., 2016). Indigo blue dyes have been important in driving economics through the production of dyer's weed, *Isatis tinctoria* and its economic rival, *Indigofera tinctoria*, which emerged with the expansion of European trade routes to India (Asiaticus, 1912; Sandberg, 1989; Clark et al., 1993). The presence of blue in nature inspired artists such as Michaelangelo, Albrecht Duerer, Gauguin, Picasso, and van Gough who used blue pigments like lapis lazuli or organic pigments. Blue is also used to represent important religious symbols such as the Hindu deity Lord Krishna (Blurton, 1993; Prabhupada Bhaktivedanta Swami, 2013, Bhagat Gita English edition) and the Virgin Mary (Pastoureau, 2001; Heller, 2008; Fallon, 2014). The relative scarcity of blue available in natural pigments likely fueled our fascination with the preferred color of many humans.

Color perception in humans is enabled by our trichromatic visual system containing photoreceptors maximally absorbing radiation of wavelengths about 421, 530, and 559 nm (blue, green, and red). Our visual system compares the responses of these photoreceptors by means of an opponent system (Hurvich, 1981; Koenderink, 2010), which can also be influenced by a variety of ecological and contextual effects (Palmer et al., 2013). A more detailed explanation of comparative color vision is provided in the second part of this work, but first we address how color as a trait has typically been employed for classifying plants as a baseline to our current understanding. To better understand how we or other animals use spectral information requires care and consideration of the context of how color may work differently depending upon the observers and their visual experience (Palmer et al., 2013; Kemp et al., 2015).

While much of human history is surrounded by blue skies and waters, this fascination may nevertheless have also emerged from its perceived rarity in the biological world (Goethe, 1810: "Farbenlehre"). A blue flower was a central symbol of inspiration for the Romanticism movement in Europe (Novalis, 1802) and remains important in contemporary Western art (Gage, 1999). In fact, flowers perceived by humans as being blue (**Figure 1**) are infrequent, constituting less than 10% of the nearly 300,000 known species of flowering plants (Lee, 2010). Blue flowers are also reported to be phylogenetically restricted, occurring in only 372 out of 14,038 genera of angiosperms worldwide, and in 53 out of 406 plant families (Gottsberger and Gottlieb, 1981). Considering available data in the newly extended international plant trait database "TRY database" (Kattge et al., 2020), an overall 772 of 10,437 (7%) species are classified as being "blue" flowers, with other human-perceived colors being more frequent (**Figure 2** and **Supplementary Figure 1**). Interestingly, blue is far more common among biotically-pollinated flowers in the TRY database than among wind-pollinated flowers, where blue colors are almost non-existent, although in general wind pollinated flowers lack salient colors (**Figure 2**). These data suggest that exploring the rarity of blue flowers requires a deeper understanding of how animals perceive these colors. Flower color plays an important role in the taxonomy of plants by helping differentiate between single species (Linnaeus, 1735, 1755, 1785) as well as in the ecology of plants by attracting pollinators (Chittka and Menzel, 1992; Shrestha et al., 2013; Ohashi et al., 2015; LeCroy et al., 2021). In addition, other factors can also be at play, as recent evidence suggests that increased pollinator competition may also promote convergence toward the most preferred colors (Shrestha et al., 2019a; Tai et al., 2020), which is discussed in depth below. Accordingly, in harsher conditions, with less competition, higher divergence of flower colors is observed (Dalrymple et al., 2020).

Surprisingly, it is not only humans for which short wavelength blue coloration has a special allure: honeybees (Morawetz et al., 2013), bumblebees (Gumbert, 2000), and stingless bees (Dyer et al., 2016a) have innate preferences for short wavelength blue colors. This leads to the important question of why blue is not more frequently observed among flower colors in nature, and, indeed, whether this impression of blue scarcity is correct. This multidisciplinary research question draws attention to fields as diverse as the biochemistry of floral pigments, land-use management, and biodiversity research, as well as the biogeography of available resources in different environments, and how humans and pollinators perceive color. We discuss these factors (biochemistry of blue pigment evolution and pollinators, developmental and environmental controls of blue, nutrient availability and plant diversity with respect to blue flowering species, land use intensity as a driver of flower color richness, water availability, and drought stress) below to provide bridges into our comparative understanding of flower color evolution, and how blue flowers might be an important biomarker of complex factors influencing biodiversity.

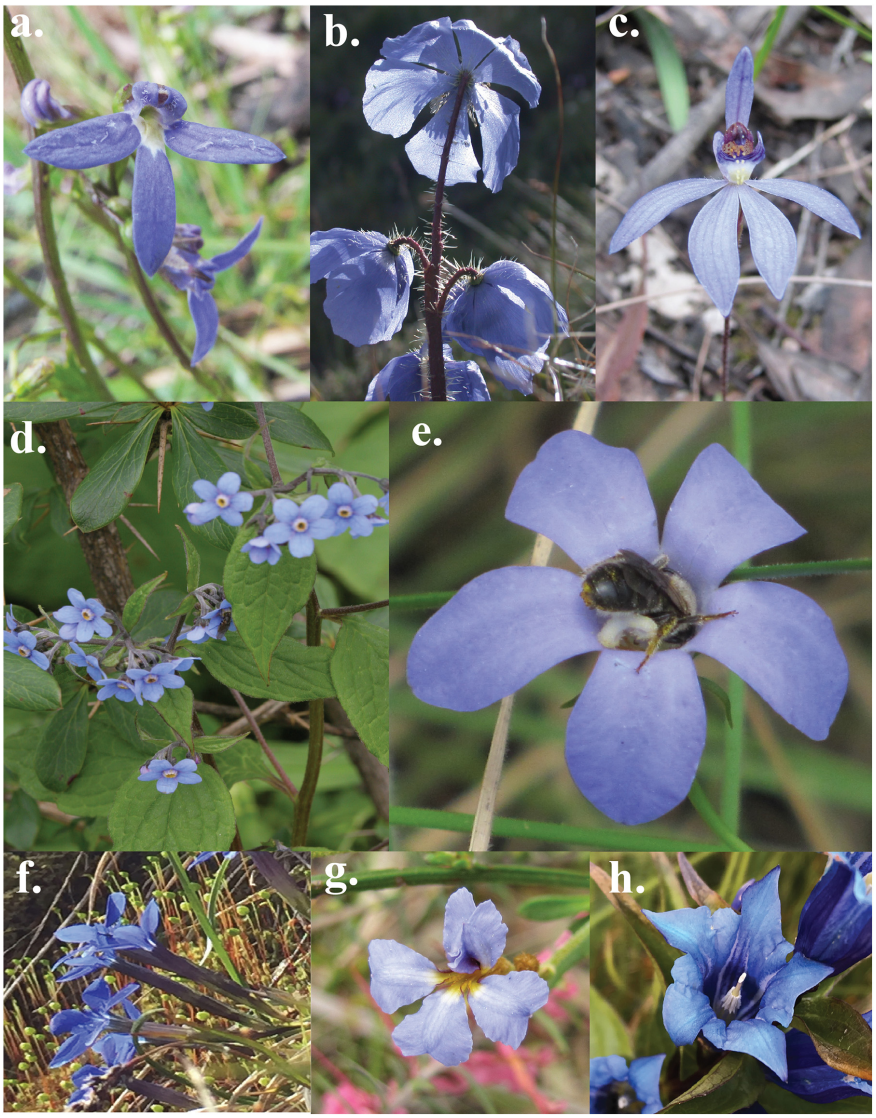


FIGURE 1 | Example of some flowers perceived as blue by a human observer: **(a)** *Lobelia rhombifolia*, **(b)** *Meconopsis horridula*, **(c)** *Cyanicula caerulea*, **(d)** *Hackelia uncinata*, **(e)** *Wahlenbergia gloriosa*, **(f)** *Gentiana bavarica*, **(g)** *Dampiera stricta*, and **(h)** *Gentiana asclepiadea* (Image Credit: Anke Jentsch, Mani Shrestha).

BIOCHEMISTRY OF BLUE PIGMENT EVOLUTION AND POLLINATORS

Flower colors are mainly determined by the chemical structure of anthocyanins (ancient Greek for "blue flower"), a group of flavonoids (Grotewold, 2006; Katsumoto et al., 2007; Tanaka et al., 2008). Although the synthetic pathway for anthocyanin can yield several alternative forms (Lee, 2007), the majority of blue flowers contain delphinidin-based anthocyanins (Honda and Saito, 2002). The pH in the vacuole, where anthocyanins are localized, can also alter the color of anthocyanins, with blue colors produced in a weakly acidic or neutral cellular environment (Goto and Kondo, 1991). Stacking of co-occurring pigments like flavones or flavonols with anthocyanins or the formation of a complex with metal ions (Fe 3^{+}, Mg 2^{+}, 2 $Ca2^{+}$) can be key elements for the production of blue flowers (Kondo et al., 1992; Yoshida et al., 2003; Shiono et al., 2005; Shoji et al., 2007). It takes a complicated chemical pathway to generate blue flower color, where six anthocyanins together with six co-occurring molecules form a ring around two central metal ions. With a multitude of potential mechanisms for modifying anthocyanin pigments to produce blue colors (Lee, 2007), it remains unclear what inhibits plant species from expressing blue flower coloration (Yoshida et al., 2009). This is especially true when blue coloration may also be achieved via structural coloration (Vignolini et al., 2015; Moyroud et al., 2017), and/or a mixture of pigment and structural colors (van der Kooi and Stavenga, 2019).

Anthocyanins comprise three major types: pelargonidin (generally red), cyanidin (typically magenta or blue depending on pH), and delphinidin (generally blue) (Mol et al., 1996;

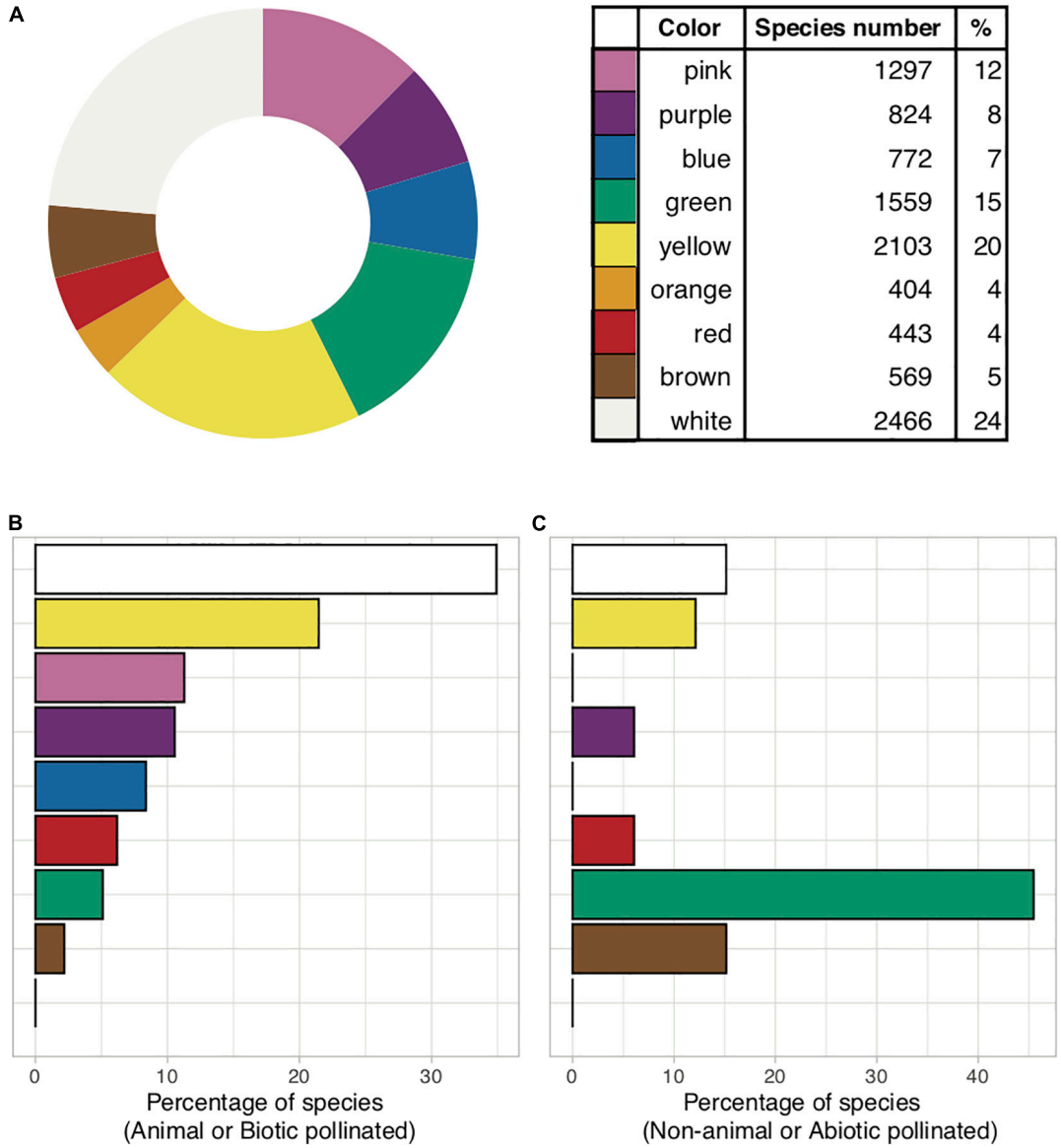


FIGURE 2 | (A) Global flower color frequency based on human visual perception (n = 10,437 species; data source: Kattge et al., 2020, https://www.try-db.org/TryWeb/). **(B)** Flower color frequency in animal pollinated species (n = 275) where less than 10% are blue. **(C)** Non-animal pollinated species (n = 33), where blue does not occur. All wind-pollinated angiosperms are secondarily wind-pollinated and thus the color of these flowers might be relicts from a previous animal pollinated period. **(B,C)** are limited to European data only. R code available in **Supplementary Appendix 1**.

Davies, 2004). These pigments play an important role attracting fauna for pollination, seed dispersal, protection against stress, and signaling (reviewed by Koes et al., 2005). Research has shown that bird-pollinated flowers are much more likely to contain pelargonidin and much less likely to contain delphinidin than other flowers (Scogin, 1988; Davies, 2004), whereas insect-visited plants may contain flavonoid, delphinidin, cyanidin, and carotinoid pigmentation (Davies, 2004; Samanta et al., 2011). Plant pigments and pollinator groups are classified according to classical pollination syndromes as perceived by humans (Davies, 2004). Furthermore, Smith and Rausher (2011) reported shifting production of delphinidin to pelargonidin in *Ipomea gesnerioide*, which shows that the bird-pollinated flowers are most frequently evolved from bee-pollinated plants. Thus, floral color can evolve through quantitative variation in the production of several types of pigments (Davies, 2004; Kellenberger et al., 2019), leading to complex floral reflectance spectra (Davies, 2004; van der Kooi et al., 2016). Recent work also shows that the potential color effect of pigments can be further modified by the distribution of pigment cells in the structure of the flower petal due to optical effects (van der Kooi et al., 2016), suggesting that flowers potentially have a number of biologically plausible mechanisms to tune or modulate color signals.

DEVELOPMENTAL AND ENVIRONMENTAL CONTROLS

Anthocyanin production can be induced by both developmental and environmental controls (Farzad et al., 2003). For instance, temperature or ultraviolet light intensity can influence the floral anthocyanin content (Mol et al., 1996; Lu et al., 2009) and therefore the intensity of flower colors. Additionally, resource restriction such as cold temperature, a lack of nitrogen (Do and Cormier, 1991; Rajendran et al., 1992; Bongue-Bartelsman and Phillips, 1995) or phosphorus, exposure to lower pH (Suzuki, 1995), stress such as wounding (Ferreres et al., 1997), or pathogen infection (Dixon et al., 1994) may increase anthocyanin production (Chalker-Scott, 1999). In addition, certain nutrients, in particular aluminum Al^{3+}, combined with a low soil pH can induce a color change from purple to blue in some plant species (Chalker-Scott, 1999). Plant species can accumulate only limited kinds of anthocyanins and therefore there may be limits on the production of some flower colors by the expression of a specific set of biosynthetic genes (Katsumoto et al., 2007). As a result, in some plant families like roses, carnations and chrysanthemum no blue flowers occur naturally due to the lack of a key enzyme in the synthesis of delphinidin. In comparison, blue flowers occur in those taxa of angiosperms that have a higher proportion of herbaceous species, which are mainly insect pollinated. Blue flowers are thought to be rare in early diverging lineages, which are rather associated with wind pollination (**Figure 2**), and more frequent in derived groups like Asteridae, Commelinidae and some clades of Liliidae like *Linum* sections *Linum* and *Dasylinum* (McDill et al., 2009). Even with sophisticated genetic engineering techniques, it was extremely difficult to modify the colors of "white" roses to reflect blue wavelengths of light (Ogata et al., 2005; Tanaka et al., 2009), a challenge that would have considerable commercial benefits due to human aesthetics and color preferences, and perhaps requiring changes to popular poems and tunes using the verse "*Les bluets sont bleus, les roses sont roses*," immortalized in Victor Hugo's novel "Les Misérables" (Hugo, 1863, p. 97), and its English equivalent, "Roses are Red, Violets are Blue."

NUTRIENT AVAILABILITY AND PLANT DIVERSITY

Finding patterns that drive diversity in plant systems and in plant communities is a major issue in plant ecology. Emerging questions include how productivity affects species richness and trait distribution in plant communities. The humped-back model (HBM) (Grime, 1973) suggests that plant species richness peaks at intermediate productivity, taking above-ground biomass as a proxy for annual net primary productivity. This diversity peak is driven by two opposing processes (**Figure 3**, Fraser et al., 2015). In unproductive and disturbed ecosystems with low plant biomass, species richness is limited by either abiotic stress, such as insufficient water and mineral nutrients, or high levels of disturbance-induced biomass removal, which few species are able to tolerate. In contrast, in the low disturbance and productive conditions that generate high plant biomass, exclusion by a small number of highly competitive species is hypothesized to constrain species richness. Other mechanisms that may explain the unimodal relationship between species richness and productivity include disturbance (Connell, 1978; Jentsch and White, 2019), evolutionary history, and dispersal limitation (Taylor, 1990; Zobel and Pärtel, 2008).

Additional factors including nutrient availability (**Figure 4**) (Bedford et al., 1999; Fraser et al., 2015) and habitat disturbance (Grime, 1973; Jentsch and White, 2019) may also influence flower color and plant species diversity (**Figure 4**), although at present causal mechanisms remain largely unknown. With increasing species richness, the percentage of blue flowering species has been reported to increase (Ostler and Harper, 1978; Schemske and Bierzychudek, 2007). In mesotrophic grasslands, blue flowers are reported to be absent from the most species-poor communities (Warren and Billington, 2005). Generally, co-flowering species increase the flower color complementarity and diversification of a community (Lázaro et al., 2009; de Jager et al., 2011; Makino and Yokoyama, 2015; Losapio et al., 2017). It is thought to be potentially advantageous to stand out from flowers of competitive species (Makino and Yokoyama, 2015) and also from the background against which flowers are viewed (Bukovac et al., 2017b) in order to attract pollinators, although continental surveys (Chittka and Menzel, 1992; Dyer et al., 2012; Bukovac et al., 2017a) and community studies (Kantsa et al., 2017, 2018; Shrestha et al., 2019b) reveal that plant flowers frequently converge to preferred signals of particular pollinators. This suggests that either high plant diversity may drive increased flower color diversity as a means of attracting specialist pollinators (Weiner et al., 2011; Mesgaran et al., 2017), and/or that blue flower color is viable environmentally in places that promote or require high diversity.

LAND USE INTENSITY AND FLOWER COLOR DIVERSITY

Diversity is often driven by land-use intensity (Collins et al., 1998). Increasing land-use intensity results in fewer species as well as lower flower color species richness (Warren and Billington, 2005; Binkenstein et al., 2013) (**Figures 5**, **6**). Increasing land-use intensity, measured in an index combining fertilization, grazing, and mowing, produced a shift in flower colors from less frequent blue toward mainly white flowering species as perceived by a human with normal color vision (Binkenstein et al., 2013). Specific effects of fertilization, grazing, or mowing could not be identified, and likely require more powerful models that incorporate the diversity of pollinator mediated influences.

In Scandinavia and the rest of Northern Europe all the way down to Bavaria in Southern Germany, the traditional, extensively cultivated flowering meadows are found in the lowlands and in the mountains, although in the lowland's meadows increasing habitation is changing the environment in complex and unknown ways. Cultivated flower meadows seem to have an unusually high percentage of species with blue flowers

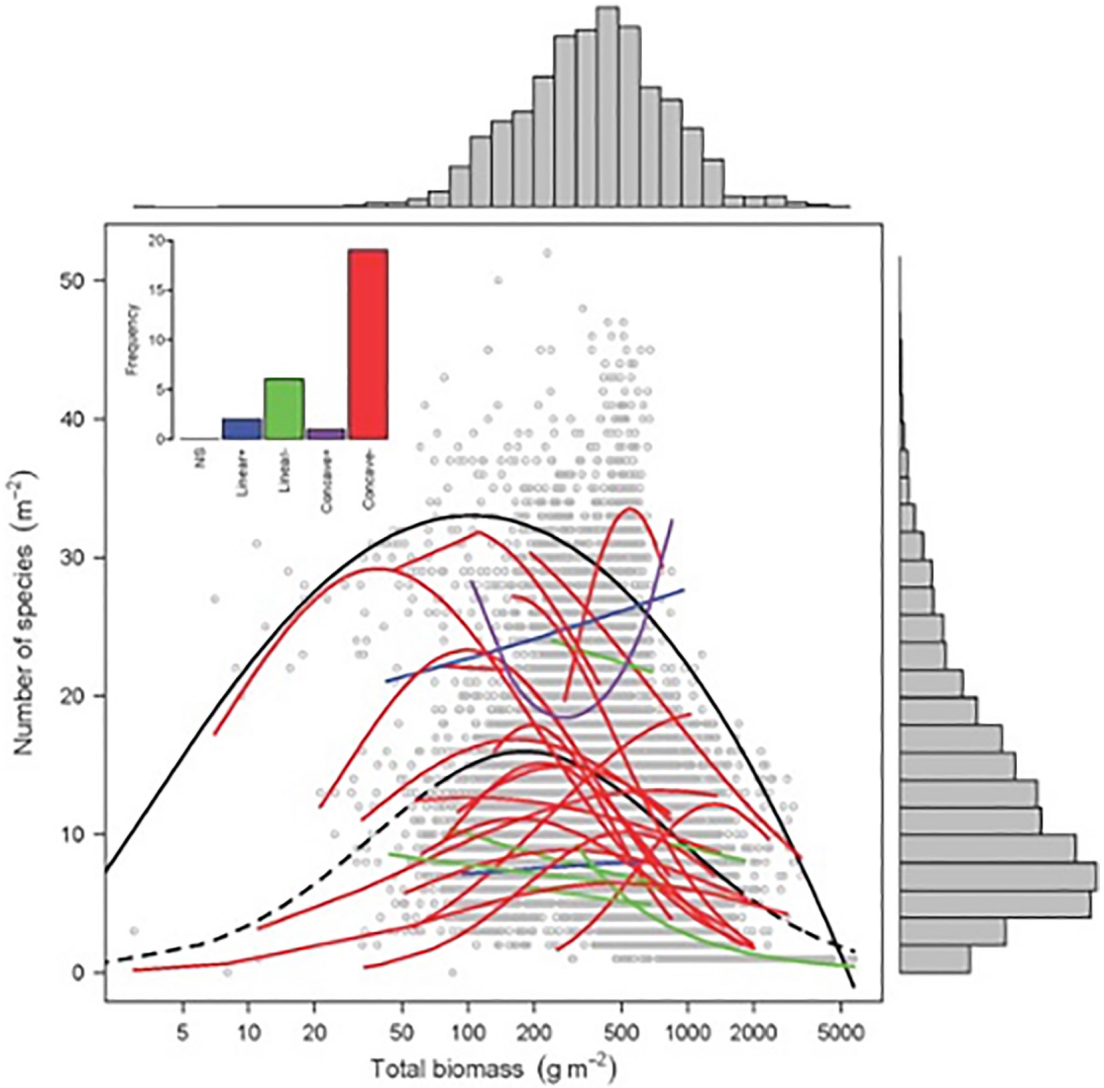


FIGURE 3 | Biomass–richness relationship for 28 grasslands across the world. Black solid line: significant quantile regression (95%) of overall relationship ($P < 0.001$; $n = 9631$ quadrats). Colored lines: significant GLM regressions (Poisson or quasi-Poisson), with N ranging from 128 to 894 (Source: Fraser et al., 2015). Reprinted with permission from AAAS, permission no: 4976850603684.

(see, e.g., Stabbetorp and Endrestøl, 2011; Austad et al., 2015), and reduced meadows cause the demise of species possessing blue flowers such as *Dracocephalum ryschiana*, *Campanula barbata*, *Campanula rotundifolia*, *Jasione montana*, *Polygala serpyllifolia*, *Polygala amarella*, *Gentiana pneumonante*, *Gentiana nivalis* (and several other *Gentiana* species), *Hepatica nobilis* [*Anemone hepatica*], and *Viola hiarta* (as well as the more common violets). These meadows are threatened by climate warming, fertilization, and increased mowing frequency (Berauer et al., 2019) or from transition to modern farmland, development, or simply falling into fallow and eventually being taken over by forest. The decrease in small flowering species has also been associated with increase in nitrogen and phosphorus, which causes competition from large grasses and herbs (e.g., Stevens et al., 2004). These flowering species are typically found on unfertilized hayfields and pastures, especially on calcareous soils, which facilitates mineral uptake. In addition, such soils are often rich in minerals such as potassium, magnesium, and iron.

Some works suggest that many rare poorly competitive species with blue flowers may lose out to competition from larger plants. For example, blue-flowered species, such as violets (*Viola* sp.) (Jeffrey and Pigott, 1973; Maskell et al., 2010) and *C. rotundifolia* (Stevens et al., 2004; Maskell et al., 2010), are shown to be lost when the nitrogen and/or phosphorus content increases. Some small blue-flowered species may fail even from the actual increased phosphorus and/or nitrogen, as is reported for *J. montana* (Tyler, 1992). Also, the increased deposition of atmospheric nitrogen, which is quite low, may be a threat to small blue-flowered grassland species (see Stevens et al., 2004, 2011). Of other environmental changes, increased soil acidity from precipitation may also affect these low-growing calciphiles, as reported for *H. nobilis* (or *A. hepatica*) (Tyler et al., 2002).

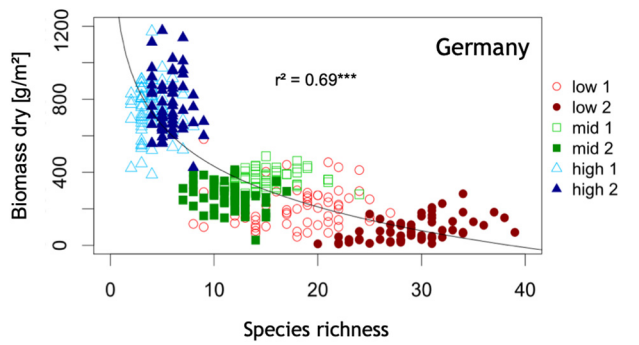


FIGURE 4 | Plant species richness as a function above ground biomass production: Highest community plant species richness is associated with lowest community productivity at this half of the productivity gradient. Low = site of low productivity, mid = sites of intermediate productivity, high = sites of high productivity. Data shown here are based on 364 plots of 1 m × 1 m area organized in six systematic grids across a local productivity gradient in mesic temperate grassland close to Bayreuth University in Germany, part of the Herbaceous Diversity Network HERBDIVNET (see details in Fraser et al., 2015). ***$p < 0.001$.

Ekstam and Forshed (1992) provided a comprehensive list of nitrogen tolerance in plants and whether they are weak competitors. They note, for example, *Viola*, *Euphrasia*, *Polygala*, and some *Veronica* species to be particularly weak competitors with very low tolerance to nitrogen increase. Thus, many species with blue flowers show some evidence of being susceptible to land-use changes, especially since they are expected to be restricted to low nitrogen and low phosphorus but high micronutrient (calcium and other) environments. It is also worth asking whether the presence of blue flower may act as biomarkers of healthy land/ecosystem that have intense land use pattern with uses of different fertilizers.

WATER AVAILABILITY AND DROUGHT STRESS

Other factors such as water stress may influence the occurrence of blue flower color. Blue flowers of *Lysimachia arvensis* perform better in terms of seedling mass or reproductive age in dry environments compared to a red morph (Arista et al., 2013). Schemske and Bierzychudek (2001) found a similar effect in *Linanthus parryae*, in that blue morphs produced more seeds than white morphs in years of low spring precipitation, possibly linked to bee preference. Since important pollinators like bees prefer blue hues, this suggests that in more stressful environments, selection might favor blue floral colors if possible, to provide resilience in the face of resource restrictions as to help attract presumably scarcer bee pollinators, as pollinator limitation is a major factor in plant reproductive success (Burd, 1994; Bennett et al., 2020; Giejsztowt et al., 2020). However, few

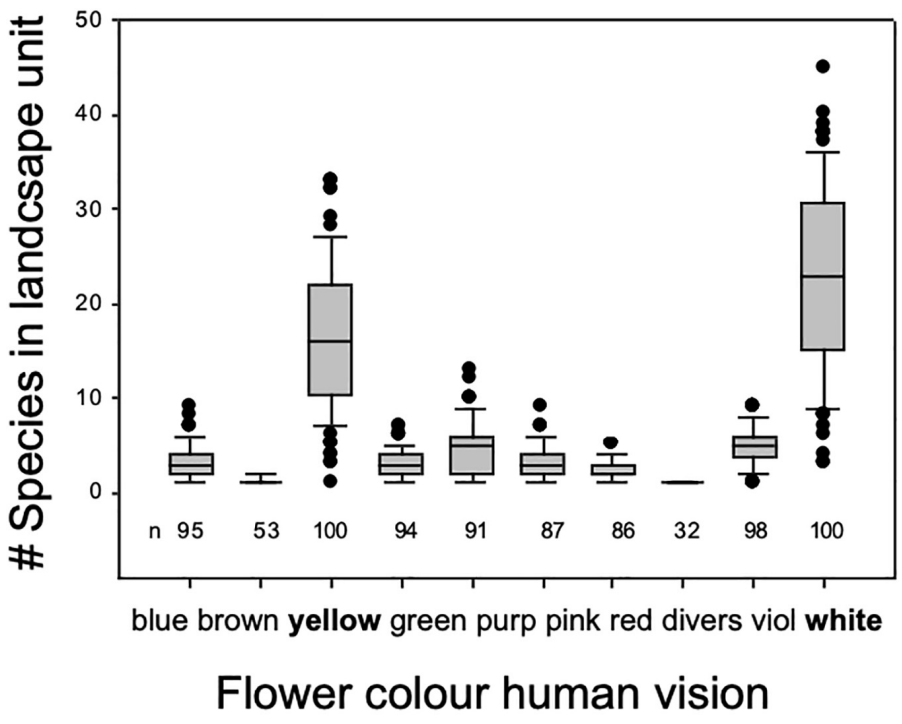


FIGURE 5 | Number of plant species displaying flower color by 10 categories as sensed by human vision in a typical semi-natural, montaneous landscape in central Europe. Data are based on 100 plots of 100 m × 100 m systematically arranged with a 4 km × 4 km landscape unit a calcareous bedrock harboring mesic grasslands, small deciduous forests, hedges, rocky outcrops, partly grazed, partly mown, partly logged in the Franconian Swiss, Bavaria, Southern Germany regions. Most flowering forbs in this diver's cultural landscape are perceived yellow or white by humans.

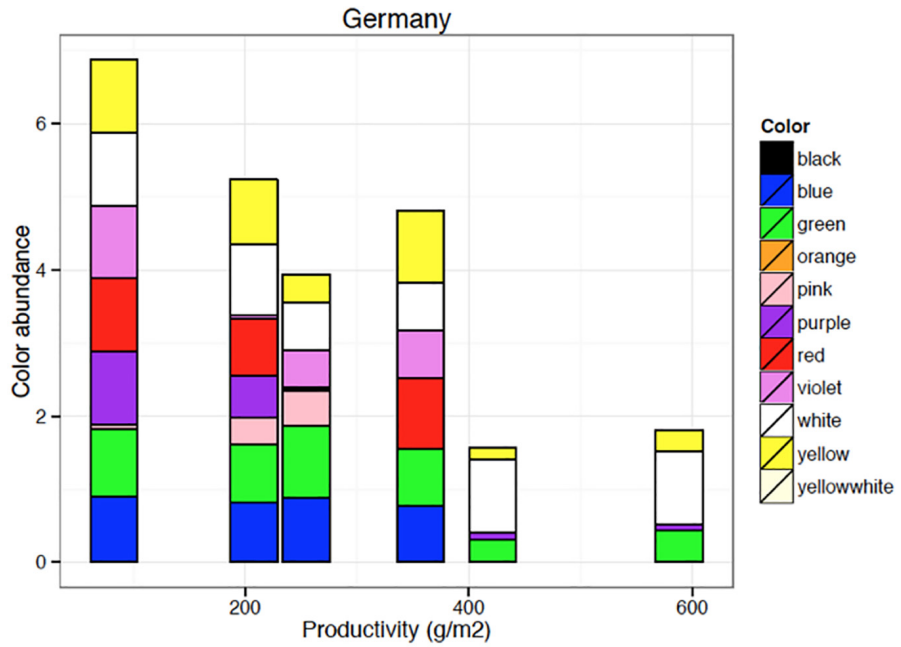


FIGURE 6 | Flower color abundance of plant species growing on sites of various productivity. Blue and red flowering species (as seen by human vision) disappear from the plant communities with increasing site productivity. Data shown here are based on 364 plots of 1 m × 1 m area organized in six systematic grids across a local productivity gradient in mesic temperate grassland close to Bayreuth University in Germany, part of the Herbaceous Diversity Network HERBDIVNET (see details in Fraser et al., 2015).

studies have considered pollinator perception in such analyses, and below we attempt to synthesize available evidence to bridge the potentially contributing abiotic and biotic factors that might influence flower coloration. In this regard, recent work evaluating potential abiotic or biotic factors for the Australian continent reveals that both may be important, but biotic factors modeled with appropriate bee pollinator color space appear to be the main evolutionary driver of flower coloration (Dalrymple et al., 2020).

VISION OF BLUE ACROSS SPECIES

Human eyes are sensitive to light which lies in a very small region of the electromagnetic spectrum labeled "visible light." This "visible light" corresponds to radiation with a wavelength range of about 400–700 nanometers (nm) which we perceive as a range of colors from violet through red. Three types of wavelength selective photoreceptors are responsible for our normal color vision. The blue (or S for Short) photoreceptor with a peak sensitivity at 421 nm, green (or M for Medium) photoreceptor at 530 nm, and red (or L for Long) photoreceptors at 559 nm (Stockman and Sharpe, 2000). Notable in the context of the discussion above (**Figures 5**, **6**), humans do not actually have dedicated color photoreceptors for colors perceived as yellow. Our color perception is enabled by opponent color processing where red and green cone photoreceptor responses are processed in an opponent fashion with responses from our blue cone photoreceptor to generate the sensation of yellow, which is why we most typically see yellow and blue as opposite in representations like a color wheel (Hurvich, 1981). In a similar way to the blue preference in many adults discussed above, color perception is influenced by environmental effects (Palmer et al., 2013). This partially explains why color screening tests are done for driving a car, as, due to the complexity of human color vision, some individuals cannot tell the color difference between green and red, but still can discriminate some other colors (Backhaus et al., 1998; Stockman and Sharpe, 2000). Once we appreciate this point, it becomes questionable to what extent human color vision is appropriate for assessing flower colors that evolved for different animals, and a purpose of this manuscript is to provide a bridge between different ways of interpreting spectral data from flowers. **Figure 7** shows, for example, the comparative visual system of different animals known to interact with flowering plants in a biologically relevant way, showing that what is "blue" is likely to be a perceptual dimension of a particular observer in many different ways. Some insect pollinators like butterflies and moths show evidence of a high level of diversity (Chen et al., 2013), even between genders of a single species (Arikawa et al., 2005). This variability is partly due to the complex genetics of the group where ommatidial types can be stochastically distributed in their eyes as shown for species belonging to two separate groups (Perry et al., 2016). For example, the trichromatic hawkmoth (*Manduca sexta*) shows a preference for blue (Goyret et al., 2008), other lepidopteran species like *Papilio xuthus* have six photoreceptor classes implicated in color vision that are also potentially subject to other light tuning factors that enable very difference color capabilities (Arikawa et al., 1999; Arikawa, 2003). Taken

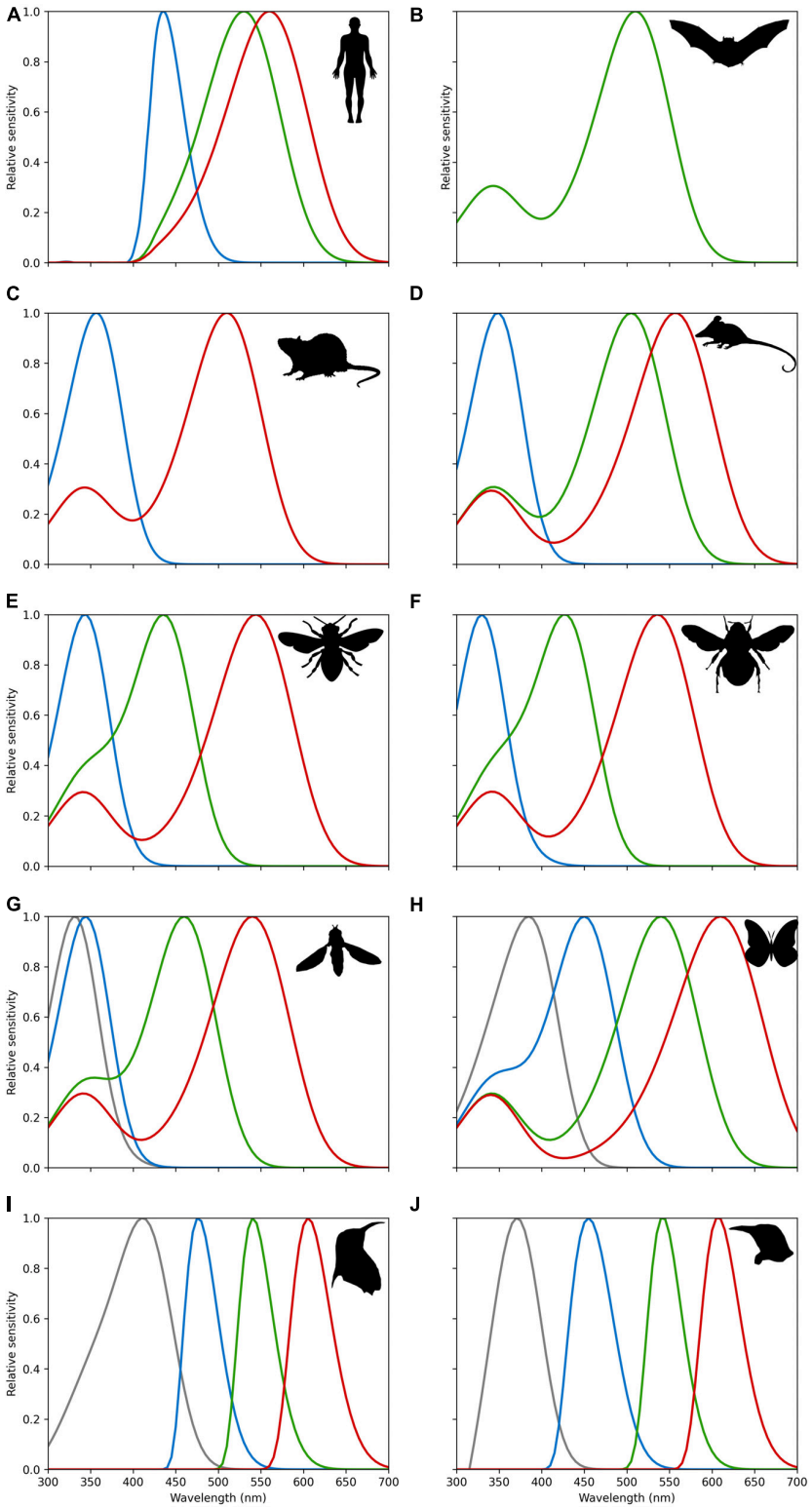


FIGURE 7 | Spectral sensitivities of photoreceptors for **(A)** Trichromatic humans (*Homo sapiens*) (Dartnall et al., 1983; Stockman and Sharpe, 2000) as a point of comparison, and various animals know to visit flowers; **(B)** monochromatic flower bat (*Glossophaga soricine*) that senses short wavelength light via a secondary beta-band peak (Winter et al., 2003); **(C)** dichromatic mouse (*Mus musculus*) (Sun et al., 1997; Wester et al., 2009); **(D)** trichromatic Australian honey possum (*Tarsipes rostratus*) (Arrese et al., 2002); **(E)** trichromatic honeybee (*Apis mellifera*) (Peitsch et al., 1992; Briscoe and Chittka, 2001); **(F)** trichromatic bumble bee

(Continued)

FIGURE 7 | Continued
(*Bombus terrestris*) (Peitsch et al., 1992; Briscoe and Chittka, 2001); **(G)** tetrachromatic drone fly (*Eristalis tenax*) (Lunau, 2014); **(H)** tetrachromatic swallow tail butterfly (*Papilio aegeus*) (Matić, 1983); **(I)** tetrachromatic violet sensitive bird (Endler and Mielke, 2005; Hart and Hunt, 2007); **(J)** tetrachromatic ultraviolet sensitive bird (Endler and Mielke, 2005; Hart and Hunt, 2007). All sensitivities were modeled by implementing the Stavenga et al.'s (1993) vitamin A1 visual template namogram to enable easy comparison. It is known in humans and avians that ocular filtering modifies light reaching photoreceptors (Douglas and Marshall, 1999). For bird data, we thus also implemented generic ocular filtering functions representing typical ultraviolet and violet sensitive birds as proposed by Endler and Mielke (2005). For humans' cornea and lens transmittance for a close relative primate species for which data are available (*Macaca fascicularis*). *Macaca fascicularis* data were extracted from data reported by Douglas and Marshall (1999). Line colors represent approximate region of the electromagnetic spectrum as typically perceived by humans. Relative sensitivity values for all species are provided in an electronic tabular form as **Supplementary Material** to enable future comparative research. Fly vision spectral sensitivity is mediated by green-sensitive photoreceptors coupled with UV-sensitive antennal pigments; thus, the spectral sensitivity of the four classes of R7/8 photoreceptors is likely to be relatively narrower and partially overlapping than the prediction here presented from the vitamin A1 template (Lunau, 2014). Detailed sensitivity data are available as **Supplementary Appendix 2**.

together with available evidence for some other important flower visitors (**Figure 7**), this shows that great care is required when considering how a flower color may appear to its biologically relevant pollinator.

PLANT–POLLINATOR INTERACTIONS

The main adaptive advantage promoting the evolution of specific flower colors is to capture the attention of preferred pollinators (Chittka and Menzel, 1992; Bukovac et al., 2017a), while also avoiding attention from other flower visitors that might only seek to rob flower rewards (Lunau et al., 2011; Camargo et al., 2019; Dyer and Shrestha, 2019). Darwin (1877) postulated that innate preferences might allow flower visitors to more easily find rewarding flowers, and indeed different insect clades have different innate color preferences, which likely represent phylogenetic adaptations of emerging insects foraging to first find rewarding flowers (Giurfa et al., 1995; Lunau and Maier, 1995; Raine and Chittka, 2005; Raine et al., 2006; Dyer et al., 2007, 2016a, 2019; Raine and Chittka, 2007; Ings et al., 2009; Morawetz et al., 2013; van der Kooi et al., 2019). Bees, for instance, have phylogenetically conserved trichromatic color vision with UV-, blue-, and green-receptors (e.g., **Figures 7E,F**), centering around 350, 440, and 540 nm, respectively (Chittka, 1996; Briscoe and Chittka, 2001; Dyer et al., 2012). Where two spectrally different photoreceptors overlap (e.g., around 400 nm in the blue end of the spectrum for bees), color resolution and learning is the strongest (von Helversen, 1972; Peitsch et al., 1992) and could promote color preferences (Menzel et al., 1974). For example, the common blue flowers in the order *Delphinium* are preferred by bees compared to relatively rarer white flower morphs, and the blue color appears to allow bees to see the flower better and thus results in the collection of more nectar rewards per unit time (Waser and Price, 1983). Indeed, experiments that artificially manipulate the color of such flowers with blue paint observe an increase in the efficiency with which both bumblebees and birds visit flower morphs (Waser and Price, 1985). In a similar way, wild-type flowers of *Antirrhinum majus* appear blue to bumblebees, and are both preferred and processed faster than genetically modified mutant flowers of bee-white appearance (Dyer et al., 2007). Nevertheless, it has also to be considered that many flowers are multi-colored, and the effects of such color patterning on pollinators and their preferences for different parts of the spectrum are complex and only starting to be explored (Lunau et al., 2016). In Germany, it was reported that blue flowers more frequently presented higher reward (Giurfa et al., 1995) than alternative flower colors. However, recent work in Australia reports no evidence that any particular color among bee-pollinated flowers was associated with higher nectar rewards (Shrestha et al., 2020), and so currently there is no conclusive evidence of higher rewards being associated with blue flowers.

According to Rodríguez-Gironés and Santamaría (2004), interactions among pollinators of different types might interfere with the expression of a color preference. When both bees and birds are in the same environment with equally rewarding blue or red flowers, birds may elect to preferentially visit red flowers since bees are taking rewards from the bee-preferred blue morphs, even though birds can efficiently process either color with their visual system. Thus, the difference in color visual systems among pollinators (see **Figure 7**) can influence which plant species may be successful in different environments, and such effects can be dynamic and depend upon the variety of flower visitors in an environment (Shrestha et al., 2013; Camargo et al., 2019), and how distributions may change due to factors like climate (Hegland et al., 2009; Shrestha et al., 2018). For example, when flower-visiting flies are the only pollinators in an environment like Macquarie Island in the Southern Ocean, all flowers from a wide range of plant families have been observed to reflect colors rich in long wavelength that are preferred by flies (Lunau, 2014; Shrestha et al., 2016). This strong effect consistent with pollination syndromes was also observed in a community in South Eastern Australia where orchids that were pollinated by bees more frequently had short wavelength blue colors, while fly-pollinated flowers in the same environment were never blue (Shrestha et al., 2019a).

IN SEARCH OF BLUE: *SPECTRAL ANALYSIS AND BEE COLOR SPACE*

To explore the importance of blue flower color and considering bee pollinator color vision, it is interesting to compare reflectance spectra of plants from different latitudes and geographic regions. Datasets from Australia (Dyer et al., 2012; Shrestha et al., 2019a,b), Nepal (Shrestha et al., 2014), Norway (Arnold et al., 2009, 2010), and Brazil (Camargo et al., 2019) provide an accessible comparison, because similar data collection methods were employed in the various studies, although some abiotic

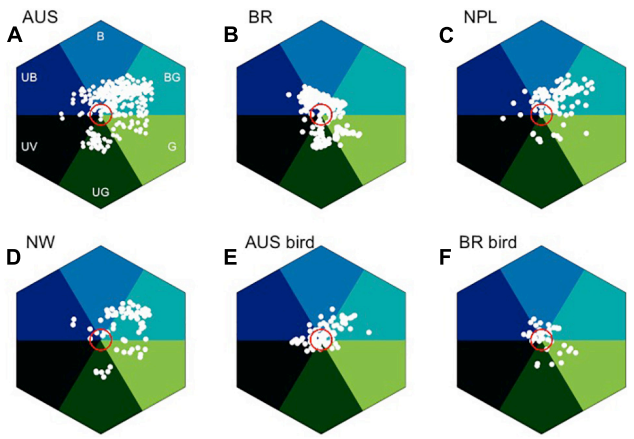


FIGURE 8 | In search of blue: bee color space for four different countries, and also considering separately bird pollinated where quality data are available. **(A)** Australian insect-pollinated flowers (AUS, *n* = 146), **(B)** Brazilian bee-pollinated flowers (BR, *n* = 245), **(C)** Nepal (NPL, *n* = 107), **(D)** Norway (NW, *n* = 77), **(E)** Australian bird-pollinated flowers (*n* = 92), and **(F)** Brazilian hummingbird-pollinated flowers (*n* = 39). Red circle is 0.11 hexagon unit which shows the achromatic region where bees are predicted to be poor at detecting colors against a foliage background, showing that bird-pollinated flowers are more frequently achromatic considering bee color perception. Hex sector values are available in **Supplementary Appendix 3**.

factors including background and/or light can vary with increasing altitude (Niu et al., 2020). The floral spectra from these studies were expressed in a bee color space, a geometrical interpretation allowing for modeling colors as perceived by an animal observer (**Figure 8**) (Kelber et al., 2003; Renoult et al., 2017); this model is implemented using a visual namogram (Stavenga et al., 1993) to model spectral sensitivity functions (**Figure 7**) for typical bee photoreceptors (350, 440, and 540 nm), using standard foliage background and open midday illumination (Judd et al., 1964), enabling the calculation of a spectral locus for the main flower color for each plant species (**Figure 8**).

In the bee color space proposed by Chittka (1992), flower loci for the Australian (**Figure 8A**), Brazilian (**Figure 8B**), Nepalese (**Figure 8C**), and Norwegian (**Figure 8D**) species are rare or non-existent in the "UV" category, but all other categories of the color hexagon do contain a significantly higher number of floral color signals. In the Australian data, where it has been possible to identify insect- or bird-pollinated flowers (Shrestha et al., 2013), very few insect-pollinated flowers have loci near the achromatic center of the bee color space that represents leaf foliage (**Figure 8A**). In contrast, many bird-pollinated flowers appear achromatic to bees (**Figure 8D**), and thus are difficult to be discriminated from the background and harder to detect by a bee observer. This difference results from the frequent evolution of red floral signals among bird-pollinated flowers (Shrestha et al., 2013; Burd et al., 2014), which tend to have spectral patterns that only weakly modulate bee photoreceptors (Lunau et al., 2011). It has been argued that the highly saturated red of bird-pollinated flowers has evolved to make flowers less apparent to bees and thus serving as bee avoidance mechanism (Lunau et al., 2011; Camargo et al., 2019; Coimbra et al., 2020). However, the spectral evidence could also be compatible with direct selection by birds, with the effect on bee perception arising as a by-product. For example, in **Figure 8E**, we see that in addition to the approximately 30% of flowers within the achromatic region of color space, the majority of bird-pollinated flowers can be visually

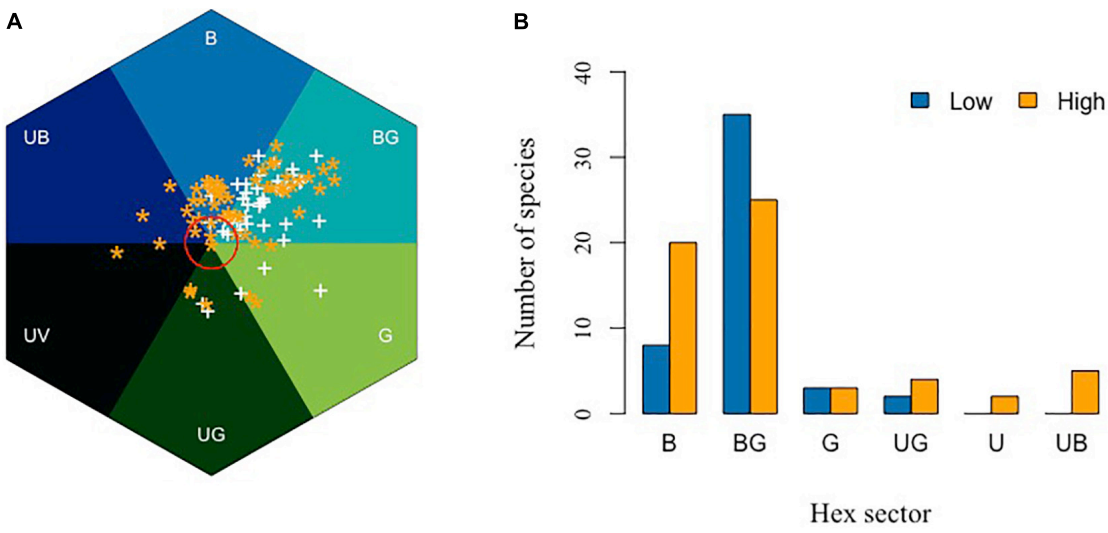


FIGURE 9 | In search of blue in different elevation. **(A)** Bee color space for low (900–2000 m, white symbol "+") and high (2700–4100 m, orange asterisk "*") altitude of Nepal. Red circle is 0.11 hexagon unit which shows the achromatic region where bees are predicted to be poor at detecting colors against a foliage background. **(B)** Number of Himalayan plant species classified in the various hexagon categories of bee color space. Flower colors are not equally distributed across color sectors (Chi-square = 6.77, df = 1, $P = 0.009$), with a higher number of blue flowers being observed at higher altitudes ($z = 2.76$, $p < 0.05$). Data are available in **Supplementary Appendix 3**.

detected by bees. Thus, more research on flower coloration using direct measurement of selection in field settings is required to dissect complex competing hypotheses of bee avoidance vs. selective pressure to evolve colors birds optimally process.

Color is a perception resulting from the particular way in which a brain processes visual information; therefore, it is important to use a model enabling for the interpretation of opponent processing to fully understand how color signaling is perceived (Chittka, 1992). Of the six sectors of bee color space, the most frequent flower color signals from Australia, Nepal, and Norway are in the "Blue-Green" (BG) category of color space (**Figure 8**), which is known to have high frequency of bee pollinated flowers (Chittka et al., 1994). In the Brazilian environment, the "Blue" (B) sector contains the most insect-pollinated flowers, followed by "Blue-Green" flowers (**Figure 8B**). A key reason for this short-wavelength preference is likely to be that by choosing blue colors, bees are able to reduce the effect of noisy signals resulting from long wavelength reflecting surfaces that are commonly found in nature (Bukovac et al., 2017b). The outstanding question then is why flowers categorized as being blue (B) as compared to blue-green (BG) are less frequent in the studied environments when considering biologically relevant bee observers (**Figure 8**)? A plausible explanation is that for pollinators to select a preferred color, they must first detect the color, which is a complex visual problem in natural environments (Bukovac et al., 2017a,b), a task which in bees is predominantly modulated by the achromatic processing channel of the long wavelength sensitive green photoreceptor (Giurfa et al., 1996; Dyer et al., 2008, 2016a,b; Wertlen et al., 2008; Skorupski and Chittka, 2011). Thus, flowers that from a bee's perspective conform to these twin visual requirements need to modulate both blue and green photoreceptors. However, such colors should not modulate the UV receptor, as that would result in an achromatic stimulus that is difficult to discriminate from the background (Kevan et al., 1996, 2001; Waser and Chittka, 1998; Spaethe et al., 2001), which has been shown to be the case with

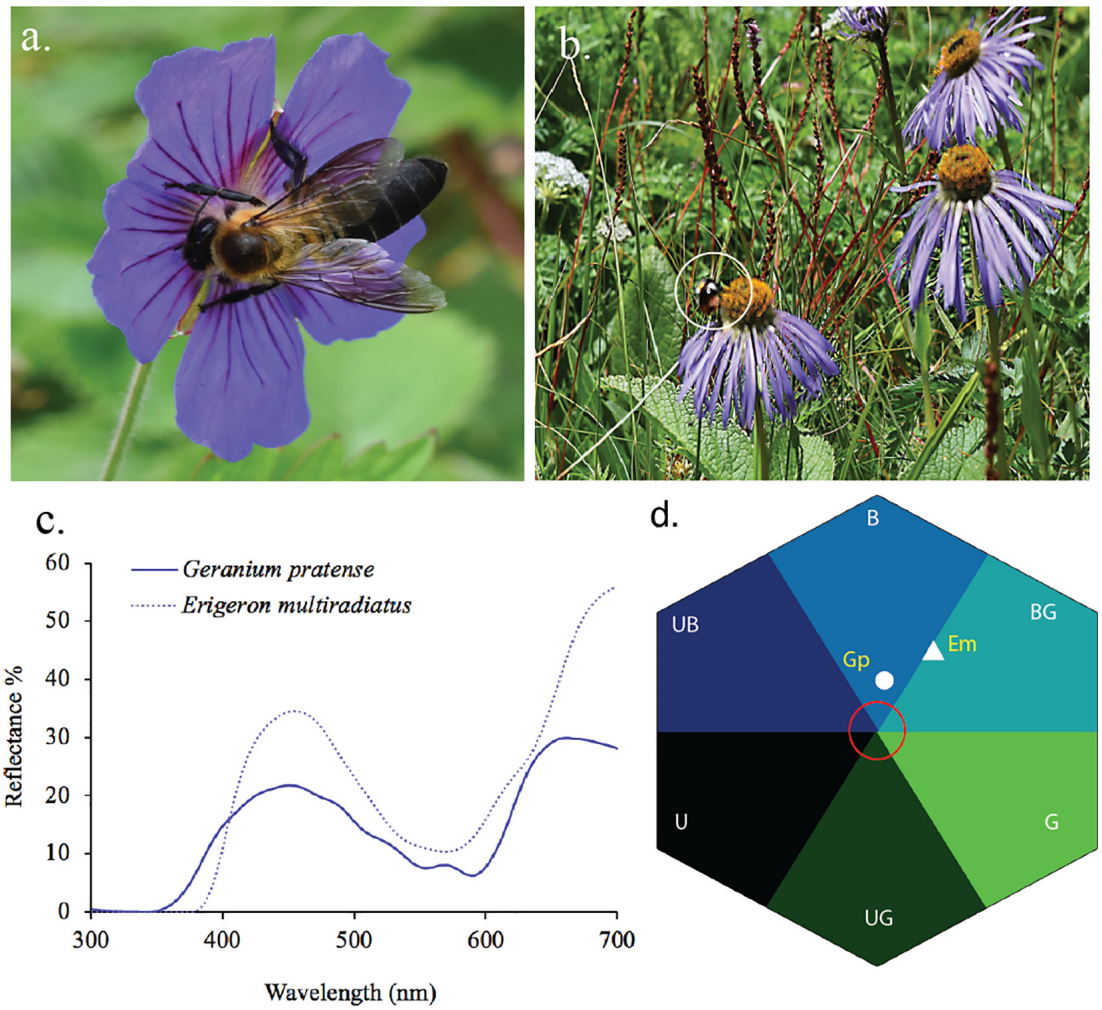


FIGURE 10 | Blue flower and its pollinators from Nepalese Himalaya. **(a)** *Geranium pratense* (GP) visited by Himalayan native bees *Apis laboriosa*, **(b)** *Eriogeron multiradiatus* (Em) visited by native bumble bees (*Bombus* sp. shown in white circle), **(c)** reflectance spectra of the two aforementioned species, and **(d)** reflectance spectra converted into bee hexagon color space. In Nepal Himalaya, native bees are present up to 4200 m a.s.l. (Thapa, 2000) and bumblebees are available/found up to 5000 m a.s.l. including some part of Sikkim Himalaya (Williams et al., 2010; Shrestha et al., 2014; Streinzer et al., 2019).

gene-modified flowers (Dyer et al., 2007). This effect may appear counterintuitive to a human observer as we process brightness or intensity cues as a dimension of our color perception, but such a capacity is due to specialized neural circuitry in the primate visual system. In fact, evidence from honeybee studies shows that these pollinators do not reliably process brightness cues (Ng et al., 2018). Therefore, flower color loci lying in the "Blue-Green" (BG) sector of bee color space have spectra that bees both easily detect and innately prefer. This BG sector of bee color space represents the loci of human white flowers, which are also observed by our eye to be most frequent in nature (**Figure 5**). Many Australian and Brazilian bird-pollinated flowers also exist in this bee blue-green (BG) color sector (**Figure 8E**), but the flowers tend to be clustered toward the center of color space (**Figure 8F**). In addition to the importance of bee pollination, abiotic conditions may also be responsible for the predominance of "Blue" (B) flowers in the Brazilian environment (**Figure 8B**). In fact, the studied flower community corresponds to highly diverse mountain vegetation subjected to seasonally dry climate, high irradiance, and acidic soils with low nutrients content and high aluminum saturation (Silveira et al., 2016).

ELEVATIONAL GRADIENTS

Flowers in higher elevations may also need to have more efficient color signaling to maximize the chance of attracting pollinators. Bees are efficient pollinators but do, in general, tend to be more frequent at lower elevations, while Diptera and Lepidoptera are more frequently observed at higher elevations (Arnold et al., 2009). Arnold et al. (2009) hypothesized that flower colors should shift from being more frequent in the B and UB categories of bee color space at low elevations, toward colors that reflect longer wavelengths at higher altitude due to a change in pollinator distributions, although their subsequent analyses of data up to 1600 m a.s.l. revealed no significant difference in flower coloration along an altitudinal gradient in Norway. In New Zealand, a high proportion of flowers in high altitudes displays colors perceived as being white by a human observer. However, recent research shows that while many mountain flowers in New Zealand are indeed white to a human observer, such flowers actually absorb UV and are thus highly chromatic for bees, lying in the BG category of bee color space, which is consistent with a preference for blue colors and reliable signal detections by bee pollinators (Bischoff et al., 2013). In the Himalayan mountains, it has been shown that floral colors were significantly more diverse at a high elevation (3000–4100 m a.s.l.) subalpine zone than in the subtropical zone (900–2000 m a.s.l.) of Nepal (Shrestha et al., 2014). **Figure 9A** shows the relative distribution of the flower colors from Nepal plotted considering the two altitudinal ranges, and shows that there is a significant shift toward shorter wavelength blue colors toward higher altitudes (**Figure 9B**).

Previous authors have observed and reported, based on human color vision, that with increasing altitude there appeared to be more blue flowers (Weevers, 1952), and by using modern pollinator observer models, it is possible to quantify evidence for such an effect (**Figure 9**). This observation leads to two lines of inquiry for the future to understand the significance of blue flower frequency with increasing altitude, including (i) are such observations consistent with the evidence from lower altitude reports that blue flowers are more frequent in harsher (e.g., drier; Schemske and Bierzychudek, 2001; Arista et al., 2013) environments and/or (ii) is it the presence of specialist high altitude bee pollinators (**Figure 10**) that promotes blue flower coloration? These hypotheses may not necessarily be mutually exclusive, as harsher conditions for plants likely also mean harsher conditions for insect pollinators. This, in turn, may create competition for pollinators, as plants are often pollinator limited in harsher conditions, potentially leading to a need for flowering plants to optimally advertise with the colors preferred by bees. Much work is required to understand what factors might lead to changes in flower color in different environments, and hopefully this review on fragmentary blue serves as a useful tool for bridging our understanding between how plants science and botany has classically thought of blue flower colors, and what the perception of such colors likely means for biologically relevant pollinators like bees.

CONCLUSION

Our human color vision (**Figure 7**) enables seeing a small fragment of the electromagnetic spectrum. Through history humans have been attracted by the blue color which has been used to decorate items of economic or ritual value and inspired poets and artists. Our synthesis review shows that human blue flowers color is a rare color in nature (**Figure 2A** and **Supplementary Figure 1**), as reported by previous authors (Gottsberger and Gottlieb, 1981; Lee, 2010), and that the pathways enabling for the production of a blue coloration suggest that plant flower colors are potentially important biomarkers of changing environmental conditions like nutrient availability. However, human color vision is not an objective tool for the evaluation of color as it is perception highly variable between different individuals and context; for example, the famous blue/gold dress dilemma recently received widespread international attention and shows that human color vision sees the same stimuli as very different colors depending upon context (Winkler et al., 2015). Flowers did not evolve under the pressure of human color vision, so modeling pollinator vision with established techniques may provide a less biased insight into color mediated interaction between animal observers and plants that enable biotic pollination. Indeed, when considering harsh environments like high altitude in the Himalayan mountains, we observe that short wavelength blue flowers do indeed become more frequent (**Figure 9**), suggesting that biotic pollination is a key factor that must always be considered in mapping flower biodiversity. Thus, whenever considering biological factors influencing flowers color signaling, or including abiotic factors reported on by researchers, care must be taken as to what observer is most relevant to a particular question, and how different observers may need to be considered to understand how and why blue flower colors exist in complex natural environments.

AUTHOR CONTRIBUTIONS

All authors contributed equally based on their expertise in each section of this review manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.618203/full#supplementary-material

Supplementary Figure 1 | Global flower color frequency across species based on the human visual system

Supplementary Appendix 1 | R code for **Figure 1**.

Supplementary Appendix 2 | Sensitivity data.

Supplementary Appendix 3 | Hexagon sector data and R code.

REFERENCES

Arikawa, K. (2003). Spectral organization of the eye of a butterfly, *Papilio*. *J. Comp. Physiol. A* 189, 791–800. doi: 10.1007/s00359-003-0454-7

Arikawa, K., Scholten, D. G. W., Kinoshita, M., and Stavenga, D. G. (1999). Tuning of photoreceptor spectral sensitivities by red and yellow pigments in the butterfly *Papilio xuthus*. *Zool. Sci.* 16, 17–24. doi: 10.2108/zsj.16.17

Arikawa, K., Wakakuwa, M., Qiu, X., Kurasawa, M., and Stavenga, D. G. (2005). Sexual dimorphism of short-wavelength photoreceptors in the small white butterfly, *Pieris rapae* crucivora. *J. Neurosci.* 25, 5935–5942. doi: 10.1523/JNEUROSCI.1364-05.2005

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Arnold, S. E. J., Faruq, S., Savolainen, V., McOwan, P. W., and Chittka, L. (2010). FReD: the floral reflectance database—a web portal for analyses of flower colour. *PLoS One* 5:e14287. doi: 10.1371/journal.pone.0014287

Arnold, S. E. J., Savolainen, V., and Chittka, L. (2009). Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. *Arthropod. Plant. Interact.* 3, 27–43. doi: 10.1007/s11829-009-9056-9

Arrese, C. A., Hart, N. S., Thomas, N., Beazley, L. D., and Shand, J. (2002). Trichromacy in australian marsupials. *Curr. Biol.* 12, 657–660. doi: 10.1016/S0960-9822(02)00772-8

Asiaticus. (1912). The rise and fall of the indigo industry in India. *Econ. J.* 22, 237–247. doi: 10.2307/2221777

Austad, I., Hauge, L., and Hamre, L. N. (2015). *Håndbok I Registering av Slåttemark (Semi-Naturlig Eng) I Sogn of Fjordane*. Rapport 04/15. Norway: Høgskulen i Sogn og Fjordane [In Norwegian]. .

Backhaus, W. G. K., Kliegl, R., and Werner, J. S. (1998). *Color Vision: Perspectives from Different Disciplines*. Berlin: De Gruyter.

Bedford, B. L., Walbridge, M. R., and Aldous, A. (1999). Patterns in nutrient availability and plant diversity of temperate north american wetlands. *Ecology* 80, 2151–2169. doi: 10.1890/0012-9658

Bennett, J. M., Steets, J. A., Burns, J. H., Burkle, L. A., Vamosi, J. C., Wolowski, M., et al. (2020). Land use and pollinator dependency drives global patterns of pollen limitation in the Anthropocene. *Nat Commun.* 11:3999. doi: 10.1038/s41467-020-17751-y

Berauer, B. J., Wilfahrt, P. A., Arfin-Khan, M. A. S., Eibes, P., Von Heßberg, A., Ingrisch, J., et al. (2019). Low resistance of montane and alpine grasslands to abrupt changes in temperature and precipitation regimes. *Arctic Antarct. Alp. Res.* 51, 215–231. doi: 10.1080/15230430.2019.1618116

Binkenstein, J., Renoult, J., and Schaefer, H. M. (2013). Increasing land-use intensity decreases floral colour diversity of plant communities in temperate grasslands. *Oecologia* 173, 461–471. doi: 10.1007/s00442-013-2627-6

Bischoff, M., Lord, J. M., Robertson, A. W., and Dyer, A. G. (2013). Hymenopteran pollinators as agents of selection on flower colour in the New Zealand mountains: salient chromatic signals enhance flower discrimination. *N. Z. J. Bot.* 51, 181–193. doi: 10.1080/0028825X.2013.806933

Blurton, T. R. (1993). *Hindu Art*. Cambridge, MA: Harvard University Press.

Bongue-Bartelsman, M., and Phillips, D. A. (1995). Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. *Plant Physiol. Biochem.* 33, 539–546.

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510.

Bukovac, Z., Dorin, A., Finke, V., Shrestha, M., Garcia, J., Avarguès-Weber, A., et al. (2017a). Assessing the ecological significance of bee visual detection and colour discrimination on the evolution of flower colours. *Evol. Ecol.* 31, 153–172. doi: 10.1007/s10682-016-9843-6

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017b). Why background colour matters to bees and flowers. *J. Comp. Physiol. A* 203, 1–12. doi: 10.1007/s00359-017-1175-7

Burd, M. (1994). Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Bot. Rev.* 60, 83–139. doi: 10.2307/4354221

Burd, M., Stayton, C. T., Shrestha, M., and Dyer, A. G. (2014). Distinctive convergence in Australian floral colours seen through the eyes of Australian birds. *Proc. R. Soc. B Biol. Sci.* 281:20132862. doi: 10.1098/rspb.2013.2862

Camargo, M. G. G., Lunau, K., Batalha, M. A. P. L., Brings, S., de Brito, V. L. G., Morellato, L. P. C., et al. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol.* 70, 1–9. doi: 10.1111/j.1751-1097.1999.tb01944.x

Chen, P. J., Arikawa, K., and Yang, E. C. (2013). Diversity of the photoreceptors and spectral opponency in the compound eye of the golden birdwing, *Troides aeacus* formosanus. *PLoS One* 8:e62240. doi: 10.1371/journal.pone.0062240

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L. (1996). Does bee color vision predate the evolution of flower color? *Naturwissenschaften* 83, 136–138. doi: 10.1007/BF01142181

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/BF00188925

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). UV as a component of flower reflections and the colour perception of hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Clark, R. J. H., Cooksey, C. J., Daniels, M. A. M., and Withnall, R. (1993). Indigo, woad, and Tyrian Purple: important vat dyes from antiquity to the present. *Endeavour* 17, 191–199. doi: 10.1016/0160-9327(93)9006-8

Coimbra, G., Araujo, C., Bergamo, P. J., Freitas, L., and Rodríguez-Gironés, M. A. (2020). Flower conspicuousness to bees across pollination systems: a generalized test of the bee-avoidance hypothesis. *Front. Plant Sci.* 11:558684. doi: 10.3389/fpls.2020.558684

Collins, S. L., Knapp, A. K., Briggs, J. M., Blair, J. M., and Steinauer, E. M. (1998). Modulation of Ddiversity by grazing and mowing in native tallgrass Prairie. *Science* 280, 745–747. doi: 10.1126/science.280.5364.745

Connell, J. H. (1978). Diversity in tropical rain forests and coral reefs. *Science* 173, 771–780. doi: 10.1126/science.173.3999.771

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1701–1703. doi: 10.1111/nph.16737

Dartnall, H. J. A., Bowmaker, J. K., Mollon, J. D., and Barlow, H. B. (1983). Human visual pigments: microspectrophotometric results from the eyes of seven persons. *Proc. R. Soc. London. Ser. B. Biol. Sci.* 220, 115–130. doi: 10.1098/rspb.1983.0091

Darwin, C. (1877). *The Different Forms of Flowers on Plants of the Same Species*. London: John Murray.

Davies, K. (2004). *Plant Pigments and Their Manipulation*. Hoboken, NJ: Blackwell publishing.

de Jager, M. L., Dreyer, L. L., and Ellis, A. G. (2011). Do pollinators influence the assembly of flower colours within plant communities? *Oecologia* 166, 543–553. doi: 10.1007/s00442-010-1879-7

Dixon, R. A., Harrison, M. J., and Lamb, C. J. (1994). Early events in the activation of plant defense responses. *Annu. Rev. Phytopathol.* 32, 479–501. doi: 10.1146/annurev.py.32.090194.002403

Do, C. B., and Cormier, F. (1991). Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera* L.) cell suspension. *Plant Cell Rep.* 9, 500–504. doi: 10.1007/BF00232105

Douglas, R. H., and Marshall, N. J. (1999). "A review of vertebrate and invertebrate ocular filters," in *Adaptive Mechanisms in the Ecology of Vision*, eds S. N. Archer, M. B. A. Djamgoz, E. R. Loew, J. C. Partridge, and S. Vallerg (Dordrecht: Springer), 95–162. doi: 10.1007/978-94-017-0619-3_5

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Garcia, J. E., van der Kooi, C. J., and Wong, B. B. M. M. (2019). Colour preferences of *Tetragonula carbonaria* Sm. stingless bees for colour morphs of the Australian native orchid *Caladenia carnea*. *J. Comp. Physiol. A* 205, 347–361. doi: 10.1007/s00359-019-01346-0

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016a). Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, A. G., Streinzer, M., and Garcia, J. (2016b). Flower detection and acuity of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A* 202, 629–639. doi: 10.1007/s00359-016-1107-y

Dyer, A. G., and Shrestha, M. (2019). Assessment of floral colour signals at a community through the eyes of the birds and bees. *New Phytol.* 222, 648–650. doi: 10.1111/nph.15724

Dyer, A. G., Spaethe, J., and Prack, S. (2008). Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. *J. Comp. Physiol. A* 194, 617–627. doi: 10.1007/s00359-008-0335-1

Dyer, A. G., Whitney, H. M., Arnold, S. E. J., Glover, B. J., and Chittka, L. (2007). Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of *Antirrhinum majus* flower colour. *Arthropod. Plant. Interact.* 1, 45–55. doi: 10.1007/s11829-007-9002-7

Eastaugh, N., Walsh, V., Chaplin, T., and Siddall, R. (2004). *Pigment Compendium: A Dictionary of Historical Pigments*. Oxford: Elsevier Butterworth Heinemann.

Ekstam, U., and Forshed, N. (1992). *Om Hävden Upphör. Kärlväxter Som Indi ato ch Hagmarker*. Sverige: Naturvårdsvärket.

Endler, J. A., and Mielke, P. W. (2005). Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* 86, 405–431. doi: 10.1111/j.1095-8312.2005.00540.x

Fallon, B. (2014). A (blue) nt: beyond the symbology of the colour blue. *Lit. Aesthet.* 24, 21–38.

Farzad, M., Griesbach, R., Hammond, J., Weiss, M. R., and Elmendorf, H. G. (2003). Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, *Viola cornuta* cv. Yesterday, Today and Tomorrow. *Plant Sci.* 165, 1333–1342. doi: 10.1016/j.plantsci.2003.08.001

Ferreres, F., Gil, M. I., Castaner, M., and Tomás-Barberán, F. A. (1997). Phenolic metabolites in red pigmented lettuce (*Lactuca sativa*). Changes with minimal processing and cold storage. *J. Agric. Food Chem.* 45, 4249–4254. doi: 10.1021/jf970399j

Franklin, A., Bevis, L., Ling, Y., and Hurlbert, A. (2010). Biological components of colour preference in infancy. *Dev. Sci.* 13, 346–354. doi: 10.1111/j.1467-7687.2009.00884.x

Fraser, L. H., Pither, J., Jentsch, A., Sternberg, M., Zobel, M., Askarizadeh, D., et al. (2015). Worldwide evidence of a unimodal relationship between productivity and plant species richness. *Science* 349, 302–305. doi: 10.1126/science.aab3916

Frost, R. (1920). Fragmentary Blue in Book Miscellaneous Poems to 1920.

Gage, J. (1999). *Color and Meaning: Art, Science, and Symbolism*. Oakland, CA: University of California Press.

Giejsztowt, J., Classen, A. T., and Deslippe, J. R. (2020). Climate change and invasion may synergistically affect native plant reproduction. *Ecology* 101, 2904–2913. doi: 10.1002/ecy.2913

Giurfa, M., Núñez, J., Chittka, L., and Menzel, R. (1995). Colour preferences of flower-naive honeybees. *J. Comp. Physiol. A* 177, 247–259. doi: 10.1007/bf00192415

Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178, 699–709. doi: 10.1007/BF00227381

Goethe, J. W. (1810). *To the Theory of Colors (The Farbenlehre)*.

Goto, T., and Kondo, T. (1991). Structure and molecular stacking of anthocyanins—flower color variation. *Angew. Chemie Int. Ed. English* 30, 17–33. doi: 10.1002/anie.199100171

Gottsberger, G., and Gottlieb, O. R. (1981). Blue flower pigmentation and evolutionary advancement. *Biochem. Syst. Ecol.* 9, 13–18. doi: 10.1016/0305-1978(81)90053-3

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Granger, G. W. (1952). Objectivity of colour preferences. *Nature* 170, 778–780. doi: 10.1038/170778a0

Grime, J. P. (1973). Control of species density in herbaceous vegetation. *J. Environ. Manag.* 1, 151–167.

Grotewold, E. (ed.) (2006). *The Science of Flavonoids*. New York, NY: Springer, 1–273.

Gumbert, A. (2000). Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48, 36–43. doi: 10.1007/s002650000213

Hart, N. S., and Hunt, D. M. (2007). Avian visual pigments: characteristics, spectral tuning, and evolution. *Am. Nat.* 169(Suppl. 1), S7–S26. doi: 10.1086/510141

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A.-L., and Totland, Ø (2009). How does climate warming affect plant-pollinator interactions? *Ecol. Lett.* 12, 184–195. doi: 10.1111/j.1461-0248.2008.01269.x

Heller, E. (2008). *Wie Farben Wirken*. Hamburg: Rowohlt Verlag GmbH.

Honda, T., and Saito, N. (2002). Recent progress in the chemistry of polyacylated anthocyanins as flower color pigments. *Heterocycles* 56, 633–692. doi: 10.3987/rev-01-sr(k)2

Hugo, V. (1863). *Les Misérables*. Lausanne: Imprimerie Typographique de A. Larpin.

Hurvich, L. M. (1981). *Color Vision*. Sinauer: Sunderland, MA.

Ings, T. C., Raine, N. E., and Chittka, L. (2009). A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 63, 1207–1218. doi: 10.1007/s00265-009-0731-8

Jeffrey, D. W., and Pigott, C. D. (1973). The response of grasslands on sugar-limestone in teesdale to application of phosphorus and nitrogen. *J. Ecol.* 61, 85–92. doi: 10.2307/2258918

Jentsch, A., and White, P. (2019). A theory of pulse dynamics and disturbance in ecology. *Ecology* 100:e02734. doi: 10.1002/ecy.2734

Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T., et al. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Opt. Soc. Am.* 54, 1031–1040. doi: 10.1364/JOSA.54.001031

Kantsa, A., Raguso, R. A., Dyer, A. G., Olesen, J. M., Tscheulin, T., and Petanidou, T. (2018). Disentangling the role of floral sensory stimuli in pollination networks. *Nat. Commun.* 9:1041. doi: 10.1038/s41467-018-03448-w

Kantsa, A., Raguso, R. A., Dyer, A. G., Sgardelis, S. P., Olesen, J. M., and Petanidou, T. (2017). Community-wide integration of floral colour and scent in a Mediterranean scrubland. *Nat. Ecol. Evol.* 1, 1502–1510. doi: 10.1038/s41559-017-0298-0

Katsumoto, Y., Fukuchi-Mizutani, M., Fukui, Y., Brugliera, F., Holton, T. A., Karan, M., et al. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating Delphinidin. *Plant Cell Physiol.* 48, 1589–1600. doi: 10.1093/pcp/pcm131

Kattge, J., Bönisch, G., Díaz, S., Lavorel, S., Prentice, I. C., Leadley, P., et al. (2020). TRY plant trait database–enhanced coverage and open access. *Glob. Chang. Biol.* 26, 119–188.

Kelber, A., Vorobyev, M., and Osorio, D. (2003). Animal colour vision — behavioural tests and physiological concepts. *Biol. Rev.* 78, 81–118. doi: 10.1017/S1464793102005985

Kellenberger, R. T., Byers, K. J. R. P., De Brito Francisco, R. M., Staedler, Y. M., LaFountain, A. M., Schönenberger, J., et al. (2019). Emergence of a floral colour polymorphism by pollinator-mediated overdominance. *Nat. Commun.* 10:63. doi: 10.1038/s41467-018-07936-x

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., et al. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* 185, 705–724. doi: 10.1086/681021

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1, 252. doi: 10.1016/1360-1385(96)20008-1

Kevan, P. G., Chittka, L., and Dyer, A. G. (2001). Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. *J. Exp. Biol.* 204, 2571–2580.

Koenderink, J. J. (2010). *Color for the Sciences*. Cambridge, MA: The MIT Press.

Koes, R., Verweij, W., and Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* 10, 236–242. doi: 10.1016/j.tplants.2005.03.002

Kondo, T., Yoshida, K., Nakagawa, A., Kawai, T., Tamura, H., and Goto, T. (1992). Structural basis of blue-colour development in flower petals from *Commelina* communis. *Nature* 358, 515–518. doi: 10.1038/358515a0

Lázaro, A., Lundgren, R., and Totland, Ø (2009). Co-flowering neighbors influence the diversity and identity of pollinator groups visiting plant species. *Oikos* 118, 691–702. doi: 10.1111/j.1600-0706.2008.17168.x

LeCroy, K. A., Arceo-Gómez, G., Koski, M. H., Morehouse, N. I., and Ashman, T.-L. (2021). Floral color properties of serpentine seep assemblages depend on community size and species richness. *Front. Plant Sci.* 11:602951. doi: 10.3389/fpls.2020.602951

Lee, D. (2007). *Nature's Palette: the Science of Plant Color*, Chap. Chicago, ILL. The University of Chicago Press.

Lee, D. (2010). *Nature's Palette: The Science of Plant Color*. Chicago, ILL: University of Chicago Press.

Linnaeus, C. (1735). *Systema Naturae, Sive Regna Tria Naturae Systematice Proposita Per Classes, Ordines, Genera, & Species*. Leiden: Haak, 1–12.

Linnaeus, C. (1755). *Philosophia Botanica: In Qua Explicantur Fundamenta Botanica Cum Definitionibus Partium, Exemplis Terminorum, Observationibus Rariorum, Adiectis Figuris Aeneis. Originally Published Simultaneously by R. Kiesewetter (Stockholm) and Z. Chatelain (Amsterdam)*. Vienna: Joannis Thomae Trattner.

Linnaeus, C. (1785). *Systema Vegetabilium (13th edition of Systema Naturae) [A System of Vegetables 2 vols. 1783–1785]*. Lichfield: Lichfield Botanical Society.

Losapio, G., Fortuna, M. A., Bascompte, J., Schmid, B., Michalet, R., Neumeyer, R., et al. (2017). Facilitation between plants shapes pollination networks. *bioRxiv* doi: 10.1101/161034

Lu, Y., Du, J., Tang, J., Wang, F., Zhang, J., Huang, J., et al. (2009). Environmental regulation of floral anthocyanin synthesis in *Ipomoea purpurea*. *Mol. Ecol.* 18, 3857–3871. doi: 10.1111/j.1365-294X.2009.04288.x

Lunau, K. (2014). Visual ecology of flies with particular reference to colour vision and colour preferences. *J. Comp. Physiol. A* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19. doi: 10.1007/bf00243394

Lunau, K., Konzmann, S., Bossems, J., and Harpke, D. (2016). A matter of contrast: yellow flower colour constrains style length in *Crocus* species. *PLoS ONE* 11:e0154728. doi: 10.1371/journal.pone.0154728

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Makino, T. T., and Yokoyama, J. (2015). Nonrandom composition of flower colors in a plant community: mutually different co-flowering natives and disturbance by aliens. *PLoS One* 10:e0143443. doi: 10.1371/journal.pone.0143443

Maskell, L. C., Smart, S. M., Bullock, J. M., Thompson, K., and Stevens, C. J. (2010). Nitrogen deposition causes widespread loss of species richness in British habitats. *Glob. Chang. Biol.* 16, 671–679. doi: 10.1111/j.1365-2486.2009.02022.x

Matić, T. (1983). Electrical inhibition in the retina of the butterfly Papilio - I. four spectral types of photoreceptors. *J. Comp. Physiol. A* 152, 169–182. doi: 10.1007/BF00611182

McDill, J., Repplinger, M., Simpson, B. B., and Kadereit, J. W. (2009). The phylogeny of *Linum* and *Linaceae* subfamily Linoideae, with implications for their systematics, biogeography, and evolution of heterostyly. *Syst. Bot.* 34, 386–405. doi: 10.1600/036364409788606244

McManus, I. C., Jones, A. L., and Cottrell, J. (1981). The aesthetics of colour. *Perception* 10, 651–666.

Menzel, R., Erber, J., and Masuhr, T. H. (1974). "Learning and memory in the honeybee," in *Experimental Analysis of Insect Behaviour*, ed. L. Barton-Browne (Berlin: Springer), 195–217.

Mesgaran, M. B., Bouhours, J., Lewis, M. A., and Cousens, R. D. (2017). How to be a good neighbour: facilitation and competition between two co-flowering species. *J. Theor. Biol.* 422, 72–83. doi: 10.1016/j.jtbi.2017.04.011

Mol, J., Jenkins, G., Schäfer, E., Weiss, D., and Walbot, V. (1996). Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. *Crit. Rev. Plant Sci.* 15, 525–557. doi: 10.1080/07352689609382369

Morawetz, L., Svoboda, A., Spaethe, J., and Dyer, A. G. (2013). Blue colour preference in honeybees distracts visual attention for learning closed shapes. *J. Comp. Physiol. A* 199, 817–827. doi: 10.1007/s00359-013-0843-5

Moyroud, E., Wenzel, T., Middleton, R., Rudall, P. J., Banks, H., Reed, A., et al. (2017). Disorder in convergent floral nanostructures enhances signalling to bees. *Nature* 550, 469–474. doi: 10.1038/nature24285

Ng, L., Garcia, J. E., and Dyer, A. G. (2018). Why colour is complex: evidence that bees perceive neither brightness nor green contrast in colour signal processing. *FACETS* 3, 800–817. doi: 10.1139/facets-2017-0116

Niu, Y., Stevens, M., and Sun, H. (2020). Commercial harvesting has driven the evolution of camouflage in an alpine plant. *Curr. Biol.* doi: 10.1016/j.cub.2020.10.078

Novalis (1802). *Heinrich Von Ofterdingen*. Bidhannagar: Projekt Gutenberg Self-Publishing Press.

Ogata, J., Kanno, Y., Itoh, Y., Tsugawa, H., and Suzuki, M. (2005). Anthocyanin biosynthesis in roses. *Nature* 435, 757–758. doi: 10.1038/nature435757a

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral colour change in the eyes of pollinators: testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144–1155. doi: 10.1111/1365-2435.12420

Ostler, W. K., and Harper, K. T. (1978). Floral ecology in relation to plant species diversity in the Wasatch Mountains of Utah and Idaho. *Ecology* 59, 848–861. doi: 10.2307/1938788

Ou, L.-C., Luo, M. R., Woodcock, A., and Wright, A. (2004). A study of colour emotion and colour preference. Part III: colour preference modeling. *Color Res. Appl.* 29, 381–389. doi: 10.1002/col.20047

Palmer, S. E., and Schloss, K. B. (2010). An ecological valence theory of human color preference. *PNAS* 107, 8877–8882. doi: 10.1073/pnas.0906172107

Palmer, S. E., Schloss, K. B., and Sammartino, J. (2013). Visual aesthetics and human preference. *Annu. Rev. Psychol.* 64, 77–107. doi: 10.1146/annurev-psych-120710-100504

Pastoureau, M. (2001). *Blue: The History of a Color*. trans. Markus I. Cruse. Princeton, NJ: Princeton University Press.

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Perry, M., Kinoshita, M., Saldi, G., Huo, L., Arikawa, K., and Desplan, C. (2016). Molecular logic behind the three-way stochastic choices that expand butterfly colour vision. *Nature* 535, 280–284. doi: 10.1038/nature18616

Prabhupada Bhaktivedanta Swami, A. C. (2013). *Bhagavad Gita*. Los Angeles, CA: Bhaktivedanta book trust.

Raine, N. E., and Chittka, L. (2005). Colour preferences in relation to the foraging performance and fitness of the bumblebee *Bombus terrestris*. *Uludag Bee J.* 5, 145–150.

Raine, N. E., and Chittka, L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One* 2:e556. doi: 10.1371/journal.pone.0000556

Raine, N. E., Ings, T. C., Dornhaus, A., Saleh, N., and Chittka, L. (2006). Adaptation, genetic grift, pleiotropy, and history in the evolution of bee foraging behavior. *Adv. Study Behav.* 36, 305–354. doi: 10.1016/S0065-3454(06)36007-X

Rajendran, L., Ravishankar, G. A., Venkataraman, L. V., and Prathiba, K. R. (1992). Anthocyanin production in callus cultures of *Daucuscarota* as influenced by nutrient stress and osmoticum. *Biotechnol. Lett.* 14, 707–712. doi: 10.1007/BF01021647

Renoult, J. P., Kelber, A., and Schaefer, H. M. (2017). Colour spaces in ecology and evolutionary biology. *Biol. Rev.* 92, 292–315. doi: 10.1111/brv.12230

Rodríguez-Gironés, M. A., and Santamaría, L. (2004). Why are so many bird flowers red? *PLoS Biol.* 2:e350. doi: 10.1371/journal.pbio.0020350

Samanta, A., Das, G., and Das, S. K. (2011). Roles of flavonoids in plants. *Carbon N. Y.* 100, 12–35.

Sandberg, G. (1989). *Indigo Textiles: Technique and History*. London: A & C Black, 184.

Schemske, D. W., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* 55, 1269–1282. doi: 10.1554/0014-3820(2001)055[1269:peofci]2.0.co;2

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower color in the desert annual Linanthus parryae: was wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Scogin, R. (1988). Floral anthocyanidins of bird-visited flowers. *Bot. Gaz.* 149, 437–442. doi: 10.2307/2995514

Shiono, M., Matsugaki, N., and Takeda, K. (2005). Structure of the blue cornflower pigment. *Nature* 436:791. doi: 10.1038/436791a

Shoji, K., Miki, N., Nakajima, N., Momonoi, K., Kato, C., and Yoshida, K. (2007). Perianth bottom-specific blue color development in tulip cv. murasakizuisho requires ferric ions. *Plant Cell Physiol.* 48, 243–251. doi: 10.1093/pcp/pcl060

Shrestha, M., Burd, M., Garcia, J. E., Dorin, A., and Dyer, A. G. (2019a). Colour evolution within orchids depends on whether the pollinator is a bee or a fly. *Plant Biol.* 21, 745–752. doi: 10.1111/plb.12968

Shrestha, M., Dyer, A. G., Garcia, J. E., and Burd, M. (2019b). Floral colour structure in two Australian herbaceous communities: it depends on who is looking. *Ann. Bot.* 124, 221–232. doi: 10.1093/aob/mcz043

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Shrestha, M., Garcia, J. E., Bukovac, Z., Dorin, A., and Dyer, A. G. (2018). Pollination in a new climate: assessing the potential influence of flower temperature variation on insect pollinator behaviour. *PLoS One* 13:e0200549. doi: 10.1371/journal.pone.0200549

Shrestha, M., Garcia, J. E., Burd, M., and Dyer, A. G. (2020). Australian native flower colours: does nectar reward drive bee pollinator flower preferences? *PLoS One* 15:e0226469. doi: 10.1371/journal.pone.0226469

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Silveira, F. A., Negreiros, D., Barbosa, N. P., Buisson, E., Carmo, F. F., Carstensen, D. W., et al. (2016). Ecology and evolution of plant diversity in the endangered *campo rupestre*: a neglected conservation priority. *Plant Soil* 403, 129–152. doi: 10.1007/s11104-015-2637-8

Skorupski, P., and Chittka, L. (2011). Photoreceptor processing speed and input resistance changes during light adaptation correlate with spectral class in the bumblebee, bombus impatiens. *PLoS One* 6:e25989. doi: 10.1371/journal.pone.0025989

Smith, S. D., and Rausher, M. D. (2011). Gene Loss and parallel evolution contribute to species difference in flower color. *Mol. Biol. Evol.* 28, 2799–2810. doi: 10.1093/molbev/msr109

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Splitstoser, J. C., Dillehay, T. D., Wouters, J., and Claro, A. (2016). Early pre-hispanic use of indigo blue in Peru. *Sci. Adv.* 2, e1501623. doi: 10.1126/sciadv.1501623

Stabbetorp, O. E., and Endrestøl, A. (2011). *Faglig Grunnlag for Handlingsplanen for Dragehode Dracocephalum Ruyschiana og Dragehodeglansbille Meligethes Norvegicus*. NINA Rapp. 766. Trondheim: Norsk institutt for naturforskning.

Stavenga, D. G., Smits, R. P., and Hoenders, B. J. (1993). Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vision Res.* 33, 1011–1017. doi: 10.1016/0042-6989(93)90237-Q

Stevens, C. J., Dise, N. B., Mountford, J. O., and Gowing, D. J. (2004). Impact of nitrogen deposition on the species richness of grasslands. *Science* 303, 1876–1879. doi: 10.1126/science.1094678

Stevens, C. J., Gowing, D. J. G., Wotherspoon, K. A., Alard, D., Aarrestad, P. A., Bleeker, A., et al. (2011). Addressing the impact of atmospheric nitrogen deposition on Western European grasslands. *Environ. Manage.* 48, 885–894. doi: 10.1007/s00267-011-9745-x

Stockman, A., and Sharpe, L. T. (2000). The spectral sensitivities of the middle- and long-wavelength-sensitive cones derived from measurements in observers of known genotype. *Vision Res.* 40, 1711–1737. doi: 10.1016/S0042-6989(00)00021-3

Streinzer, M., Chakravorty, J., Neumayer, J., Megu, K., Narah, J., Schmitt, T., et al. (2019). Species composition and elevational distribution of bumble bees (Hymenoptera, Apidae, *Bombus* Latreille) in the East Himalaya, Arunachal Pradesh, India. *ZooKeys* 851, 71–89. doi: 10.3897/zookeys.851.32956

Sun, H., Macke, J. P., and Nathans, J. (1997). Mechanisms of spectral tuning in the mouse green cone pigment. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8860–8865. doi: 10.1073/pnas.94.16.8860

Suzuki, M. (1995). Enhancement of anthocyanin accumulation by high osmotic stress and low ph in grape cells (*Vitis hybrids*). *J. Plant Physiol.* 147, 152–155. doi: 10.1016/S0176-1617(11)81428-8

Tai, K.-C., Shrestha, M., Dyer, A. G., Yang, E. C., and Wang, C. N. (2020). Floral color diversity: how are signals shaped by elevational gradient on the tropical–subtropical mountainous island of Taiwan? *Front. Plant Sci.* 11:582784. doi: 10.3389/fpls.2020.582784

Tanaka, Y., Brugliera, F., and Chandler, S. (2009). Recent progress of flower colour modification by biotechnology. *Int. J. Mol. Sci.* 10, 5350–5369. doi: 10.3390/ijms10125350

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Taylor, A. D. (1990). Metapopulations, dispersal, and predator-prey dynamics: an overview. *Ecology* 71, 429–433. doi: 10.2307/1940297

Teller, D. Y., Civan, A., and Bronson-castain, K. (2004). Infants' spontaneous color preferences are not due to adult-like brightness variations. *Vis. Neurosci.* 21, 397–401. doi: 10.1017/S0952523804213360

Thapa, V. K. (2000). *An Inventory of Nepal's Insects. Volume III (Hemiptera, Hymenoptera, Coleoptera & Diptera)*. Nepal: IUCN.

Tyler, G. (1992). Inability to solubilize phosphate in limestone soils-key factor controlling calcifuge habit of plants. *Plant Soil* 145, 65–70. doi: 10.1007/BF00009542

Tyler, T., Brunet, J., and Tyler, G. (2002). Sensitivity of the woodland herb *Anemone hepatica* to changing environmental conditions. *J. Veg. Sci.* 13, 207–216. doi: 10.1111/j.1654-1103.2002.tb02041.x

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B Biol. Sci.* 283:20160429. doi: 10.1098/rspb.2016.0429

van der Kooi, C. J., and Stavenga, D. G. (2019). Vividly coloured poppy flowers due to dense pigmentation and strong scattering in thin petals. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 205, 363–372. doi: 10.1007/s00359-018-01313-1

Vignolini, S., Moyroud, E., Hingant, T., Banks, H., Rudall, P. J., Steiner, U., et al. (2015). The flower of *Hibiscus trionum* is both visibly and measurably iridescent. *New Phytol.* 205, 97–101. doi: 10.1111/nph.12958

von Helversen, O. (1972). Zur spektralen Unterschiedsempfindlichkeit der Honigbiene. *J. Comp. Physiol.* 80, 439–472. doi: 10.1007/bf00696438

Warren, J., and Billington, T. (2005). Flower colour phenology in British mesotrophic grassland communities. *Grass Forage Sci.* 60, 332–336. doi: 10.1111/j.1365-2494.2005.00482.x

Waser, N., and Chittka, L. (1998). Bedazzled by flowers. *Nature* 394:835. doi: 10.1038/29657

Waser, N. M., and Price, M. V. (1983). Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302, 422–424. doi: 10.1038/302422a0

Waser, N. M., and Price, M. V. (1985). The effect of nectar guides on pollinator preference: experimental studies with a montane herb. *Oecologia* 67, 121–126. doi: 10.1007/BF00378462

Weevers, T. (1952). Flower colours and their frequency. *Acta Bot. Neerl.* 1, 81–92. doi: 10.1111/j.1438-8677.1952.tb00002.x

Weiner, C. N., Werner, M., Linsenmair, K. E., and Blüthgen, N. (2011). Land use intensity in grasslands: changes in biodiversity, species composition and specialisation in flower visitor networks. *Basic Appl. Ecol.* 12, 292–299. doi: 10.1016/j.baae.2010.08.006

Wertlen, A. M., Niggebrügge, C., Vorobyev, M., and Hempel de Ibarra, N. (2008). Detection of patches of coloured discs by bees. *J. Exp. Biol.* 211, 2101–2104. doi: 10.1242/jeb.014571

Wester, P., Stanway, R., and Pauw, A. (2009). Mice pollinate the Pagoda Lily, *Whiteheadia bifolia* (*Hyacinthaceae*) - first field observations with photographic documentation of rodent pollination in South Africa. *South African J. Bot.* 75, 713–719. doi: 10.1016/j.sajb.2009.07.005

Williams, P. H., Ito, M., Matsumura, T., and Kudo, I. (2010). The Bumblebees of the Nepal Himalaya (*Hymenoptera*: *Apidae*). *Insecta Matsumurana* 66, 115–151.

Winkler, A. D., Spillmann, L., Werner, J. S., and Webster, M. A. (2015). Asymmetries in blue-yellow color perception and in the color of "the dress.". *Curr. Biol.* 25, R547–R548. doi: 10.1016/j.cub.2015.05.004

Winter, Y., López, J., and Von Helversen, O. (2003). Ultraviolet vision in a bat. *Nature* 425, 612–614. doi: 10.1038/nature01971

Yoshida, K., Mori, M., and Kondo, T. (2009). Blue flower color development by anthocyanins: from chemical structure to cell physiology. *Nat. Prod. Rep.* 26, 884–915. doi: 10.1039/b800165k

Yoshida, K., Toyama-Kato, Y., Kameda, K., and Kondo, T. (2003). Sepal color variation of *Hydrangea macrophylla* and vacuolar ph measured with a proton-selective microelectrode. *Plant Cell Physiol.* 44, 262–268. doi: 10.1093/pcp/pcg033

Zemach, I., Chang, S., and Teller, D. Y. (2007). Infant color vision: prediction of infants' spontaneous color preferences. *Vision Res.* 47, 1368–1381. doi: 10.1016/j.visres.2006.09.024

Zobel, M., and Pärtel, M. (2008). What determines the relationship between plant diversity and habitat productivity? *Glob. Ecol. Biogeogr.* 17, 679–684. doi: 10.1111/j.1466-8238.2008.00400.x

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Geographic Mosaics of Fly Pollinators With Divergent Color Preferences Drive Landscape-Scale Structuring of Flower Color in Daisy Communities

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The striking variation in flower color across and within Angiosperm species is often attributed to divergent selection resulting from geographic mosaics of pollinators with different color preferences. Despite the importance of pollinator mosaics in driving floral divergence, the distributions of pollinators and their color preferences are seldom quantified. The extensive mass-flowering displays of annual daisy species in Namaqualand, South Africa, are characterized by striking color convergence within communities, but also color turnover within species and genera across large geographic scales. We aimed to determine whether shifts between orange and white-flowered daisy communities are driven by the innate color preferences of different pollinators or by soil color, which can potentially affect the detectability of different colored flowers. Different bee-fly pollinators dominated in both community types so that largely non-overlapping pollinator distributions were strongly associated with different flower colors. Visual modeling demonstrated that orange and white-flowered species are distinguishable in fly vision, and choice experiments demonstrated strongly divergent color preferences. We found that the dominant pollinator in orange communities has a strong spontaneous preference for orange flowers, which was not altered by conditioning. Similarly, the dominant pollinator in white communities exhibited an innate preference for white flowers. Although detectability of white flowers varied across soil types, background contrast did not alter color preferences. These findings demonstrate that landscape-level flower color turnover across Namaqua daisy communities is likely shaped by a strong qualitative geographic mosaic of bee-fly pollinators with divergent color preferences. This is an unexpected result given the classically generalist pollination phenotype of daisies. However, because of the dominance of single fly pollinator species within communities, and the virtual absence of bees as pollinators, we suggest that Namaqua daisies function as pollination specialists despite their generalist phenotypes, thus facilitating differentiation of flower color by pollinator shifts across the fly pollinator mosaic.

Keywords: Asteraceae (compositae), Diptera, flower color, geographic mosaics of pollinators, greater cape floristic region, pollinator driven divergence, sensory drive, South Africa

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INTRODUCTION

Flower color diversity is a striking feature of the angiosperms, with closely-related plant species, and populations within species, frequently varying geographically in flower color or patterning (e.g., Cooley et al., 2008; Ellis and Johnson, 2009; Newman et al., 2012; Muchhala et al., 2014; Wang et al., 2016). Spatial divergence in flower color can arise through neutral processes (Rausher, 2008; Wang et al., 2016; Koski and Galloway, 2020), or as a response to selection imposed by abiotic conditions (Koski and Ashman, 2015; Dalrymple et al., 2020) or non-pollinating flower visitors (Strauss and Whittall, 2006; de Jager and Ellis, 2014b; Kemp and Ellis, 2019). Alternately, under the pollinator-shift or Grant-Stebbins model of divergence, geographic mosaics of pollinators with different morphology or sensory systems can drive the divergence of floral traits, which may ultimately facilitate speciation (Johnson, 2006).

The broad associations between flower color and different pollinator groups offer indirect evidence that pollinators may have played a crucial role in floral color evolution (Fægri and van der Pijl, 1966; Fenster et al., 2004), while selection studies demonstrate more directly that pollinators can select on flower color (Harder and Johnson, 2009; Sletvold et al., 2016). Furthermore, geographically structured color forms of the same species (floral ecotypes), or closely related species, are frequently visited by different pollinators, providing additional evidence that pollinators are important drivers of flower color divergence (e.g., van der Niet et al., 2014; Newman et al., 2015; Kemp et al., 2019). However, one weakness of many associative studies between color and pollinator shifts is that the pollinator gradients which power floral divergence are rarely quantified independently of the focal plant taxa. Pollinator abundances are usually quantified through observations of visits to the focal flowers, with only a few studies employing independent datasets, such as pollinator distribution records (e.g., Waterman et al., 2011; van der Niet et al., 2014) or trapping surveys (e.g., Phillips et al., 2015), to quantify pollinator gradients. Consequently, it is seldom clear whether geographic associations between floral phenotype and pollinator assemblages are the evolved plant responses to underlying spatial mosaics of pollinator availability, or the result of spatially variable outcomes of competitive interactions of co-occurring flowers for pollination services (Muchhala et al., 2014).

Spatial pollinator mosaics can be qualitative, where different pollinators are either present or absent in different parts of the landscape, as envisaged by the classic Grant-Stebbins model (e.g., van der Niet et al., 2014). Pollinator-shift mediated floral divergence across qualitative gradients is most likely when plants have somewhat specialized pollination systems (Johnson, 2010). In contrast, spatial mosaics underlying divergence in generalist plants tend to be quantitative, involving subtle variation in the relative availability (density) of the same assemblage of pollinators across the landscape (Dilley et al., 2000; Gómez et al., 2009, 2014). Whether qualitative or quantitative, flower color divergence is expected across pollinator gradients when the dominant pollinators exhibit contrasting flower color preference resulting from innate differences in visual systems and sensory biases. This may frequently be the case, given the diversity of color vision systems across pollinating animals (Briscoe and Chittka, 2001; van der Kooi et al., 2021). However, associative learning, which is widespread in pollinators (Chittka et al., 1999; Dukas, 2008), can overwrite innate color preferences or interact with them in complex ways (Giurfa et al., 1995; Giurfa, 2004; Dyer et al., 2019). The resulting flexibility in flower color preferences and floral constancy could potentially reduce the strength of divergent selection on flower color across pollinator gradients (de Jager et al., 2011), or in some cases even generate divergent selection on flower color (Newman et al., 2012) if learned preferences are stable enough in time.

As detectability of flowers is dependent on contrast with background colors (e.g., leaves or soils; Bukovac et al., 2017; Koski, 2020), flower color divergence might also occur across spatial gradients in background coloration, such as geological boundaries. Thus, pollinators might favor flower colors that are more detectable in a particular environment, rather than choosing a particular color *per se*. This may be particularly important for flowers that are presented against soil backgrounds, such as in deserts (Menzel et al., 1997), where the background color is determined by spatially varying geologies. While the influence of background contrast on flower color evolution is a clear expectation of the sensory drive theory of signal evolution (Endler, 1992), it remains largely untested (Bukovac et al., 2017; Koski, 2020). Under this model, divergence in flower color should be underlain by spatial turnover in background color, but not necessarily turnover in available pollinators. The same pollinator, or pollinators with similar visual systems, could select for the most detectable flower colors across spatial gradients in background coloration, resulting in flower color divergence.

Here we focus on the striking landscape-level structuring of dominant flower colors in the spectacular spring mass-flowering displays of annual daisies in the arid Namaqualand region of the greater cape floristic region (GCFR) in South Africa (**Figure 1**). Kemp et al. (2019) recently showed convergence of dominant species within flowering daisy communities on shared flower color patterns. In addition, dominant flower color patterns shift across broad spatial scales, often across geological boundaries, and shifts usually involve multiple floral ecotypes or closely related species. Analysis of daisy (the dominant flowering family in these communities) visitation networks revealed that shared flower color patterns within and across communities are strongly associated with visitation by different bombyliid and tabanid flies (Kemp et al., 2019). This suggests that the spatial color structuring of mass flowering displays across the landscape might arise through spatially variable selection on flower color, or ecological filtering on the basis of flower color, across spatial gradients in the availability of the dominant fly pollinator species. However, little is known about distributions of these pollinators, and thus the structure of the geographic pollinator mosaic that underlies the landscape structuring of flower color.

The visual systems and color preferences of Namaqualand fly pollinators are also unknown, although one of the important bombyliid pollinators, *Megapalpus capensis* (Wiedemann), does exhibit strong behavioral responses to floral visual signals

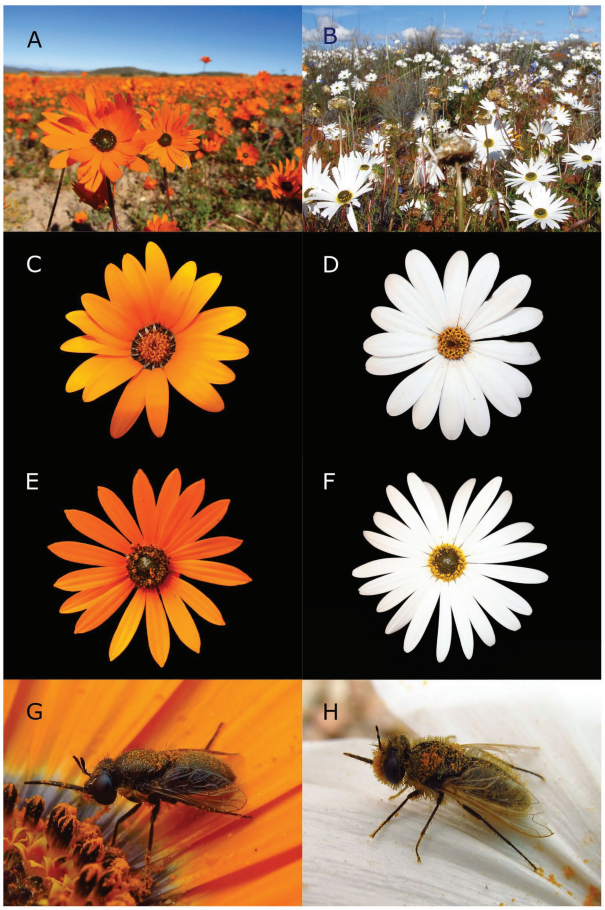


FIGURE 1 | The annual daisy species and their pollinators that characterize orange **(A)** and white **(B)** dominated communities in Namaqualand. *Dimorphotheca sinuata* **(C)** and *Dimorphotheca pluvialis* **(D)** are widespread and dominant in the Kamiesberg and Sandveld bioregions, respectively. *Ursinia cakilefolia* **(E)** and *Ursinia speciosa* **(F)** are more localized but dominate communities they occur in. The bombyliid flies, *Megapalpus capensis* **(G)** and *Corsomyza nigripes* **(H)** are dominant pollinators in the orange and white communities, respectively. The inflorescence diameter of the two *Dimorphotheca* species is on average 35 mm, and the diameter of the two *Ursinia* species is on average 31 mm. Both fly species show variation in size but are always smaller than 10 mm. Photos: JEK.

(Johnson and Midgley, 1997; de Jager and Ellis, 2012, 2014b). This knowledge gap is not surprising, given the limited understanding of color vision and preferences of flower-visiting flies generally (Lunau, 2014; Schnaitmann et al., 2020; van der Kooi et al., 2021). However, extensive variation in flower color associations (e.g., Goldblatt and Manning, 2000; Shrestha et al., 2016), photoreceptor sensitivities (van der Kooi et al., 2021), and color preferences in contexts other than flower visitation (Lunau, 2014), suggests that flower-visiting flies likely exhibit a diversity of color vision systems and innate color preferences (Lunau, 2014; van der Kooi et al., 2021), and that these may differ from those of bees (Shrestha et al., 2019).

We studied the dominant fly pollinators of focal species pairs in the daisy genera, *Dimorphotheca* and *Ursinia*, which are abundant in Namaqualand annual daisy communities dominated by white or orange flowers. We use community-level surveys to quantify the density of these pollinators across the landscape and test whether the flower color of the focal daisy species is predicted by the underlying fly density gradients. We then use visual modeling to test distinguishability and detectability of flower colors in fly vision, and cage choice experiments to test for divergent color preferences and for the influence of soil coloration on the detectability of orange and white flowers.

We investigated two potential explanations for the landscape level structuring of daisy flower color. First, if the geographic structure of flower color results from pollinator shifts across a geographic pollinator mosaic, we expect: (1) that dominant pollinator species should exhibit geographic mosaics of availability (density), (2) that pollinator species distributions should be associated with flower color distributions, and (3) that pollinators should exhibit divergent color preferences that are not flexible. Second, if the geographic structure of flower color arises through pollinator-mediated selection for detectability of flower colors across a mosaic of chromatically different soil backgrounds, we expect: (1) that flowers should be most detectable to pollinators on their local soil background and (2) that pollinator flower color preferences might be altered by the background color.

MATERIALS AND METHODS

Study System

Our study was conducted in the Namaqualand region of the Succulent Karoo, the arid component of the hyper-diverse GCFR (Born et al., 2007). We focused on two bioregions as defined by Desmet (2007): the Kamiesberg that consists of granite-gneiss dome-shaped hills, with variably grained pale soils, and the Sandveld that comprises marine-derived sands that are often red in color. In the Kamiesberg annual spring-flowering daisy displays are orange (**Figure 1A**), while the Sandveld flowering displays of annual daisies are dominated by white-flowered species (**Figure 1B**; data from Kemp et al., 2019). We focus on white-orange species pairs of *Dimorphotheca* and *Ursinia*, two distantly related Asteraceae genera (from the Calenduleae and Anthemideae tribes, respectively). *Dimorphotheca* species dominate the Namaqualand annual displays and are widespread, while *Ursinia* species are less widespread but dominate displays at some sites. Each pair comprised one species with large white ray florets [i.e., *Dimorphotheca pluvialis* (L.) Moench and *Ursinia speciosa* DC., **Figure 1**] and one with orange ray florets (i.e., *Dimorphotheca sinuata* DC. and *Ursinia cakilefolia* DC., **Figure 1**). For convenience, we refer to these as white and orange "flowered" species, respectively. While phylogenetic relationships in *Ursinia* are not resolved, *U. speciosa* and *U. cakilefolia* are members of the same clade in subgenus *Ursinia* (Magee et al., 2014). *Dimorphotheca pluvialis* and *D. sinuata* are very closely related, producing hybrids in cultivation, and the only consistent phenotypic difference between these two species is their flower

color (Norlindh, 1943). By using phylogenetically closely related species pairs, we minimize potential chemical or morphological factors that might differ between species.

Few ecological studies have been conducted on the pollinators of annual Namaqualand daisies, and work has mostly focused on the bee fly *M. capensis* that is the dominant pollinator of the hypervariable, sexually deceptive daisy, *Gorteria diffusa* (Ellis and Johnson, 2009, 2010, 2012; de Jager and Ellis, 2012, 2013, 2014a). This fly responds strongly to floral visual signals (de Jager and Ellis, 2012, 2014b) and is the dominant visitor to several species of the orange-flowered daisies which can dominate mass-flowering displays (e.g., *Dimorphotheca pinnatum* – de Waal et al., 2015, *Ursinia calenduliflora* – de Jager and Ellis, 2014b). Analysis of floral visitation networks (Kemp et al., 2019) demonstrated that, while annual Namaqualand daisies are visited by a wide array of insects as expected from their generalist phenotype, visitation is dominated by small fly species (Bombyliidae, Tabanidae), each associated with different flower color patterns across communities.

Are Flower Color Distributions in *Dimorphotheca* and *Ursinia* Overlapping?

The focal species of *Dimorphotheca* and *Ursinia* were encountered at 54 sampling sites across Namaqualand. At each site, the density of inflorescences of each species was quantified in at least twenty 1 m^2 plots along transects across a 100 × 100 m sampling area. To assess the spatial distribution of flower color, communities containing the focal species were mapped as comprising orange only, white only, or both flower colors.

Are Different Flower Colors Visited by Different Pollinator Species?

To assess whether orange and white flowers were visited by different pollinator species, we sampled 59 populations of the focal *Dimorphotheca* and *Ursinia* species in austral spring 2013–2015. We walked multiple transects through a 100 × 100 m sampling area at each site and recorded the identity and abundances of all insect species present in inflorescences of the focal plant species (mean = 690 inflorescences sampled per population, range 111–2,074). We calculated the number of individuals per flowerhead for each insect species to identify the dominant pollinator species on white and orange-flowered species, and we will focus on these dominant pollinators for the rest of the paper. We identified the dominant pollinators using two criteria: (1) species that were consistent visitors in space (i.e., present as pollinators across the most sites) and (2) species that were most frequent visitors (i.e., species that contributed the highest proportion of visits at sites).

To What Extent Do the Distribution Ranges of Dominant Pollinators Overlap?

The densities of these dominant fly pollinators were quantified using surveys at 103 sites across Namaqualand over 4 years (2013–2016) during austral spring. First, at each site (ca. 100 × 100 m) we walked multiple transects and surveyed flowers/flowerheads of all plant species on which individuals of the target pollinator species were observed, and from this, we calculated the number of insect individuals per flower/flowerhead for each plant species. This included the four focal daisy species when present, but also numerous other species of daisy, and members of other families, such as the Aizoaceae. We then estimated flower/flowerhead densities of each plant species in at least twenty 1 m^2 plots sampled on transects across the sites, and from this, we could estimate the abundances per square meter of both fly species at each site. This approach provided an estimate of availability (i.e., density) of each pollinator species independent of the phenotype of the focal plant species. To assess the spatial distribution of the identified dominant pollinators, communities were mapped as comprising *M. capensis* only, *Corsomyza nigripes* Wiedemann only, or both pollinator species.

Are Flower Color Distributions Associated With Pollinator Availability?

If pollinator species occurrence predicts flower color distributions, we expect that the dominant flower color in a community should be predicted by the availability (density m^{-2}) of pollinator species. To test this, we used the fly density and flower color data from the 54 sites where our focal plant species occurred. We coded site-level flower color, the response variable, as a binary variable (i.e., assigning orange as "1" and white as "0") because orange and white flowers did not co-occur at any sites. We ran two separate logistic regression models testing whether (1) the presence of orange flowers at a site was predicted by *M. capensis* density and (2) whether the presence of white flowers was predicted by *C. nigripes* density. As the model using *C. nigripes* densities did not converge because of complete separation (i.e., orange flowers were only present when *C. nigripes* was absent), we used Firth's bias-reduced logistic regression as implemented in the R-package logistf (Heinze and Ploner, 2018).

Are Flower Colors Detectable and Distinguishable in Fly Vision?

The reflectance spectra of the ray florets of the study species were recorded using an Ocean Optics USB4000 spectrometer, following the protocol outlined in Kemp et al. (2019). Spectra were averaged across three to five separate capitula of each species. Soil color varies strikingly across the range of our focal plant taxa, with white flowers usually presented against red marine-derived sand and orange flowers against pale yellow granite gneiss derived soil. Because background contrast can influence the detection abilities of insects (Bukovac et al., 2017), we modeled flower color in fly vision against both these background types, using a multispectral imaging approach. This was necessary because the fine granular structure of sand prevented us from reliably quantifying spectra of the sand/soil substrates using spectrometry. Four to five inflorescences of each species were imaged on each of the soil backgrounds (i.e., pale granite and red sand) using a converted and calibrated Nikon D7100 camera and an AF-S Micro Nikkor 105 mm f/2.8 lens that transmits UV wavelengths (Troscianko and Stevens, 2015). Photographs were taken in diffused sunlit conditions using a Baader UV/IR blocking filter, that transmits

from 400 to 700 nm, and a Baader U-filter optimized for transmission in the 325–369 nm range. Images were processed using the MICA toolbox (v1.22) for imageJ (v.1.4.9; Troscianko and Stevens, 2015). First images were calibrated against Spectralon 20 and 80% reflectance standards to control for lighting conditions, and then the visible and UV images were combined into aligned, normalized multispectral stacks.

Photoreceptor quantum catches were calculated for the visual system of the flower-visiting syrphid fly, *Eristalis tenax* (An et al., 2018), using the cone mapping function in the MICA toolbox. Photoreceptor sensitivities are not known for any bombyliid flies (van der Kooi et al., 2021). Quantum catches were calculated for five regions on the ray florets and soil backgrounds in each image, which were then averaged for each image. Photoreceptor excitation values for ray florets in each image were then modeled, using the color-opponent coding fly vision model of Troje (1993), against the background quantum catch for that image. Modeling followed Ohashi et al. (2015) under D65 standard daylight illumination.

The Troje (1993) model, based on data from *Lucilia* blowflies, posits that fly color vision is based on two opponency mechanisms involving two pairs of photoreceptors that allow flies to distinguish four color categories dependent on the relative excitation of these paired photoreceptors. Distinguishable color categories are represented by the four quadrants in categorical fly visual space, while colors within quadrants are not distinguishable under the Troje (1993) model. However, experiments with the flower-visiting syrphid fly, *E. tenax*, suggest that color processing is not categorical and that these flies can discriminate fine-scale differences in color within quadrants of fly color space (Hannah et al., 2019). Consequently, we used Euclidean distances from the origin of fly color space to assess the detectability of flowers on different background substrates in relation to the minimum color discrimination thresholds determined experimentally by Hannah et al. (2019). Welch's *t*-tests were used to determine whether Euclidean distances of each species from the origin of fly color space (i.e., detectability) were greater on native soils. While Hannah et al. (2019) only considered minimum discrimination distances against a background color (i.e., Euclidean distance from the origin of fly color space) and not discrimination of colors, we cautiously use these thresholds as a guide to assess discriminability of different flower colors in the color space (quantified as Euclidean distances between species centroids).

Do Pollinators Have Divergent Flower Color Preferences?

We conducted a series of choice experiments to determine flower color preferences of the dominant pollinator taxa (i.e., *M. capensis* in orange communities and *C. nigripes* in white communities) and the influence of soil background color on preferences. Wild-caught fly individuals were presented with choices between the orange and white *Dimorphotheca* and *Ursinia* species pairs separately.

To account for the potential influence of background contrast on color preference, we used the two different soil types (i.e., red sand and pale granite soil) as background for the flowers during the choice experiments. Experimental arenas contained both soil types with eight inflorescences (four orange and four white inflorescences from the same genus) placed 15 cm apart in eppendorf tubes on each soil type (i.e., 16 inflorescences in total; see **Supplementary Figure S1**). Individual flies of each pollinator species were released separately into the caged arenas, and allowed to sequentially visit inflorescences. The flower color and soil background were recorded for each visit. Trials were terminated after 5 min or when a fly had made 20 choices (median number of choices was five). Inflorescences were replaced every five trials to avoid nectar depletion. Individual flies were tested on both the *Dimorphotheca* and *Urisinia* species pairs in random order.

To test for significant differences in flower color preference between fly species, we ran a Generalized Estimating Equation (GEE) which allowed us to control for non-independence of individual fly choices. Flower color choice was set as the binomial response variable, and fly species, soil type, and their interaction were used as predictors. The model assumed an exchangeable correlation structure where the sequential choices of fly individuals are equally correlated. A binomial distribution with a logit link function was used to obtain the estimated marginal means and their 95% confidence intervals based on approximate jackknife variance estimates. Models were run separately for *Dimorphotheca* and *Ursinia* choices. Fly individuals that did not visit flowers on both soil types were excluded from the data set (*Corsomyza*: 3 of 45 trials were excluded; *Megapalpus*: 6 of 93 trials were excluded). All analyses were done in R (R Core Team, 2020) and GEEs were conducted using the geepack package (Halekoh et al., 2006).

A second set of binary choice experiments were conducted to directly assess the influence of soil background color on flower detectability. Individual insects were offered a choice between inflorescences of the same species presented on two different soil types (four inflorescences on pale soil and four on red sand). *Megapalpus capensis*, the dominant pollinator in orange communities, chose between orange *D. sinuata* flowers presented on different soils, while *C. nigripes*, the dominant pollinator in white communities, chose between white *D. pluvialis* flowers. Only the first choice of each fly was recorded, and inflorescences were replaced after every five trials. To determine whether background (i.e., soil type) influenced pollinator flower choices, Chi-square tests, expecting no differentiation in choice frequencies between soil types, were conducted separately for each fly species.

The flower color preferences of the wild-collected flies in the previous experiments could reflect either innate preferences or conditioning through experience with the particular flower color with which they associate in the field (and lack of experience with the alternative color). To distinguish between these possibilities, we conducted a series of absolute conditioning experiments (Lunau et al., 2018) to determine whether color preferences could be altered through conditioning. In the first experiment, flies were forced to feed exclusively on arrays comprising fresh inflorescences of both species of their non-preferred color (i.e., white for *M. capensis* and orange for *C. nigripes*). After an hour of conditioning, during which flies

were observed to feed copiously on both nectar and pollen, their color preference was again determined using the color choice experiment described previously. In a second experiment, *M. capensis* individuals were conditioned on the non-preferred flower color (white) for a full day before re-testing. Both *Dimorphotheca* and *Ursinia* species pairs were used for the *C. nigripes* conditioning experiment, but only *Dimorphotheca* inflorescences were available for the *M. capensis* experiments. Separate GEEs, with a binomial distribution and logit link function, were used to determine whether the flower color choice of each fly species was altered by conditioning.

RESULTS

Are Flower Color Distributions in *Dimorphotheca* and *Ursinia* Overlapping?

Flower color was entirely non-overlapping at the 54 sites where the focal plant taxa occurred (**Figures 2A,B**. The white species were only present in the Sandveld region, while the orange species were present in the Kamiesberg region (**Figure 2A**).

Are Different Flower Colors Visited by Different Pollinator Species?

Two different fly pollinators, *M. capensis* and *C. nigripes* (**Figure 1**), both from the bombyliid subfamily Mariobezziinae, were identified as the dominant pollinators of orange and white daisies, respectively. *Megapalpus capensis* contributed 45% of all observed visits on orange flowers and 1.2% on white flowers, while *C. nigripes* contributed 87% of visits to white flowers and was not observed on orange flowers (**Table 1**). Other pollinator species contributed substantially fewer visits, with bees being virtually absent (**Table 1**).

Megapalpus capensis was both the most consistent pollinator on orange flowers (present at 92% of *D. sinuata* populations and 100% of *U. cakelifolia* populations, **Table 2**), and the most frequent visitor (contributing 50% of visits on *D. sinuata* and

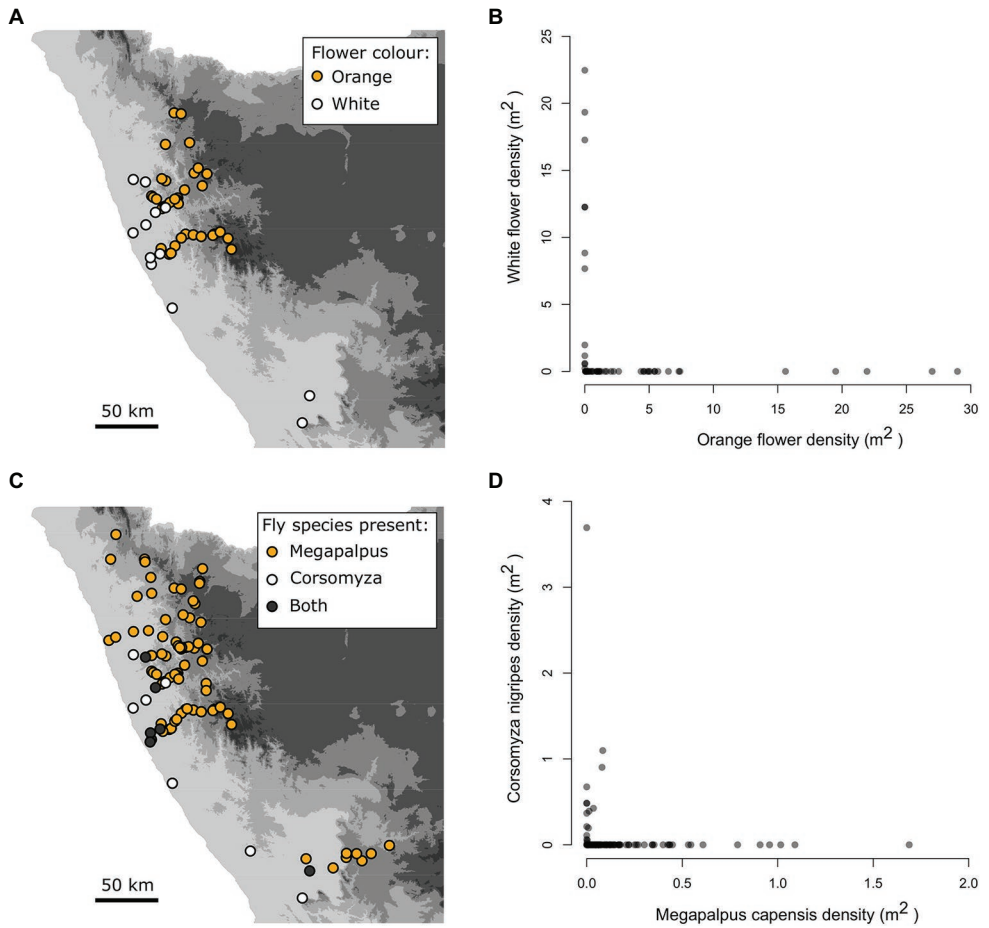


FIGURE 2 | Strongly aligned spatial gradients of flower color and pollinators of annual daisy species across the Namaqualand landscape. The non-overlapping distribution **(A)** and density relationships **(B)** of the focal orange (*Dimorphotheca sinuata*, *Ursinia cakelifolia*) and white (*Dimorphotheca pluvialis*, *Ursinia speciosa*) flowered species at 54 sites. The geographic mosaic **(C)** and density relationships **(D)** of the dominant bee-fly pollinators, *Megapalpus capensis*, and *Corsomyza nigripes*, across 103 survey sites.

37% of visits on *U. cakelifolia*). Similarly, *C. nigripes* was the most consistent pollinator on white flowers (present in 100% of *D. pluvialis* and *U. speciosa* populations) and most frequent on *D. pluvialis* (89% of visits).

To What Extent Do the Distribution Ranges of These Pollinators Overlap?

Megapalpus capensis and *C. nigripes* exhibit largely non-overlapping distributions (**Figure 2C**), and only co-occurred at 7 of 103 survey sites (i.e., 7%). *Megapalpus capensis* was present at low densities at 7 of 15 sites where *C. nigripes* occurred at relatively much higher densities (**Figure 2D**). Thus the densities of *M. capensis* and *C. nigripes* exhibit opposing geographical patterns across the Namaqualand landscape, with *C. nigripes* occurring at high density in the Sandveld and being absent from the Kamiesberg, while *M. capensis* occurs at highest densities in the Kamiesberg bioregion and is either absent or present at very low densities in the Sandveld (**Figures 2C,D**).

Are Flower Color Distributions Associated With Pollinator Availability?

The dominant flower color across sites was predicted by fly pollinator densities. High *M. capensis* densities were associated with the presence of orange daisies (df = 53, z = 2.83, p = 0.005, **Figure 3**), and high *C. nigripes* densities were associated with white daisies (df = 53, LR = 41.7, $p < 0.001$).

TABLE 1 | Proportion of total visits contributed to focal white and orange daisies by the main groups of insect pollinators.

	White flowers	Orange flowers
Corsomyza nigripes	87.3%	0%
Megapalpus capensis	1.2%	45.0%
Other flies	1.4% (4)	19.2% (8)
Hopliini beetles	3.4% (7)	25.1% (21)
Other beetles (mainly Meloidae)	6.6% (3)	10.3% (10)
Bees	0.1% (1)	0.3% (2)

Dominant fly pollinators are Megapalpus capensis for orange daisies and Corsomyza nigripes for white flowers. Data are the percentage of all insects observed on 9,334 white and 31,402 orange inflorescences during walked surveys. Numbers in brackets are the number of pollinator species/morphospecies in each group.

Are Flower Colors Detectable and Distinguishable in Fly Vision?

Spectra of white-flowered *Dimorphotheca* and *Ursinia* species were very similar, whilst the orange-flowered species did differ in the position and intensity of the secondary short wavelength (UV) peak (**Figure 4A**). Fly visual modeling suggested that the orange and white flowers can be clearly distinguished from one another, i.e., they fall in separate quadrants of the fly visual model (**Figure 4B**) and are separated by large color distances (Euclidean distances between centroids of white and orange species: *Dimorphotheca* species on red soil = 0.342, pale soil = 0.471; *Ursinia* species on red soil = 0.442, pale soil = 0.526) that are an order of magnitude larger than the minimum discrimination distances (0.021 in the p−y− quadrant, 0.059 in the p+y− quadrant) suggested by Hannah et al. (2019) for syrphids. In line with reflectance spectra differences, white-flowered species are likely indistinguishable from one another (Euclidean distance = 0.004 on red soil), while the orange-flowered *D. sinuata* and *U. cakilefolia* are more separated in fly color space (Euclidean distance = 0.114 on pale soil, **Figure 4B**) and tend to occupy different quadrants.

Euclidean distances of orange species from the origin (i.e., background) far exceeded detectability thresholds proposed by Hannah et al. (2019), while those of white species were only marginally greater than the 0.021 unit minimum detectability threshold for syrphids in the green (p−y−) quadrant of fly color space (**Figure 4C**). This suggests that, on the basis of chromatic information, all species are likely detectable by flies on the soil backgrounds, and that orange species are more detectable against both backgrounds than white species. However, capitula of the same species did not differ significantly in their detectability (i.e., distance from the origin) across the soil backgrounds (*U. speciosa*: t = 2.83, df = 2.49, p = 0.08; *U. cakilefolia*: t = 0.06, df = 4.36, p = 0.96; *D. sinuata*: t = 1.30, df = 6.62, p = 0.24; *D. pluvialis*: t = 1.00, df = 4.83, p = 0.36,

TABLE 2 | Consistency and frequency of dominant fly pollinators of the focal annual daisy species.

Color	Plant species	Number of sites	Dominant fly pollinator	Percentage of visits by dominant fly pollinator (mean ± st dev across populations)	Percentage of visits by other pollinator species (range across species)	Percentage of sites with dominant fly pollinator	Percentage of sites with other pollinator species (range across species)
White	*Dimorphotheca pluvialis*	12	*Corsomyza nigripes*	89.0 ± 11.0%	0.1–3.4%	100%	8.3–33.3%
	Ursinia speciosa	2	*Corsomyza nigripes*	16.8 ± 6.0%	2.6–36.8%	100%	50%
Orange	*Dimorphotheca sinuata*	37	*Megapalpus capensis*	49.6 ± 32.9%	0.03–7.3%	92%	2.7–40.5%
	Ursinia cakelifolia	8	*Megapalpus capensis*	37.3 ± 31.7%	0.2–15.5%	100%	12.5–75%

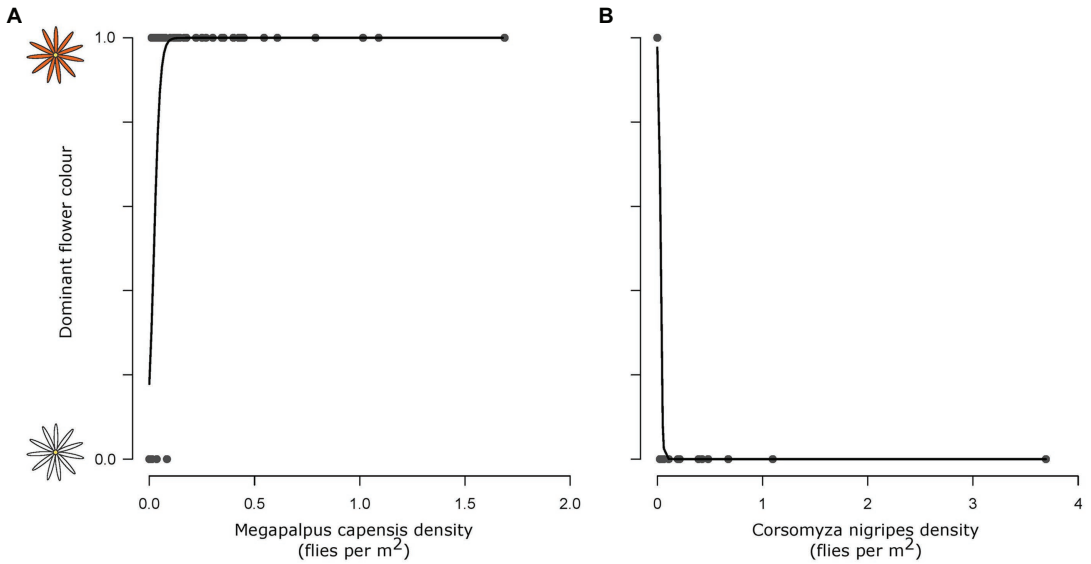


FIGURE 3 | The association between dominant flower color and pollinator species densities. *Megapalpus capensis* **(A)** and *Corsomyza nigripes* **(B)** densities predict flower color of *Dimorphotheca* and *Ursinia* species across 54 sites, where *Megapalpus capensis* is associated with orange flower dominance and *Corsomyza nigripes* is associated with white flower dominance.

Figure 4C), contrary to the expectation that species are more detectable on their native soils.

Do Pollinators Have Divergent Flower Color Preferences?

For experiments conducted using *Ursinia*, 47 *M. capensis* individuals made 482 choices and 20 *C. nigripes* individuals made 131 choices. Fly species exhibited different color preferences (Wald = 136.56, $p < 0.001$, **Figure 5**), with *M. capensis* preferring orange and *C. nigripes* preferring white, and this was not influenced by soil type (Wald = 0.16, $p = 0.69$) or the interaction between insect species and soil type (Wald = 1.10, $p = 0.30$). Similar results were found when doing the experiments using *Dimorphotheca*, with 26 *M. capensis* making 316 choices and 26 *C. nigripes* making 177 choices. Pollinators showed the same divergent color preferences as with *Ursinia* (Wald = 119.99, $p < 0.001$), which were not influenced by soil type (Wald = 0.50, $p = 0.48$) or the interaction between pollinator species and soil type (Wald = 2.91, $p = 0.09$).

Corsomyza nigripes flies more frequently chose flowers on red marine-derived soils when allowed to choose between white *D. pluvialis* flowers on the two different soil backgrounds ($\chi^2 = 9.32$, $p = 0.002$, **Figure 6**). In contrast, *M. capensis* flies showed no preference when allowed to choose between orange *D. sinuata* flowers on different soil types ($\chi^2 = 0.27$, $p = 0.60$, **Figure 6**).

Neither pollinator species exhibited altered choices after conditioning on their non-preferred flower color for an hour (*Corsomyza*: Wald = 2.96, $p = 0.09$; *Megapalpus*: Wald = 0.68, $p = 0.41$, **Figure 7**). *Megapalpus capensis* also did not show a change in preference after feeding on white flowers for a day (Wald = 1.57, $p = 0.21$).

DISCUSSION

We show that there are steep clines in the spatial turnover of flower color in the annual daisy species that dominate spring flowering displays in Namaqualand, and that change in dominant community flower color is underlain by strong gradients in the density of the dominant fly pollinators in the system. White-flowered species occupy parts of the landscape where *C. nigripes* densities are high and *M. capensis* densities low, while orange flowers dominate when *M. capensis* is present at high densities, but *C. nigripes* is absent. Visual modeling suggested that the orange and white flower colors are distinguishable in fly vision, and flower choice experiments revealed consistently strong and divergent color preferences for orange flowers by *M. capensis* and white flowers by *C. nigripes*. These spontaneous preferences could not be altered by conditioning. While we found some evidence, from choice experiments, that detectability of flowers is influenced by background soil colors, contrast between background and floral color did not alter the direction of strong color preferences. Together these lines of evidence suggest that spatial differentiation of dominant flower colors of annual daisy communities most likely results through selection or filtering imposed by divergent, apparently innate, color preferences across the largely non-overlapping distributions of dominant fly pollinators.

Color Preferences of Fly Pollinators

Color preferences have been demonstrated for many flies (Lunau, 2014), although seldom in the behavioral context of flower visitation (e.g., Campbell et al., 2010; Lunau et al., 2018; Whitehead et al., 2019). When viewed against the strength of color discrimination and preference quantified from other

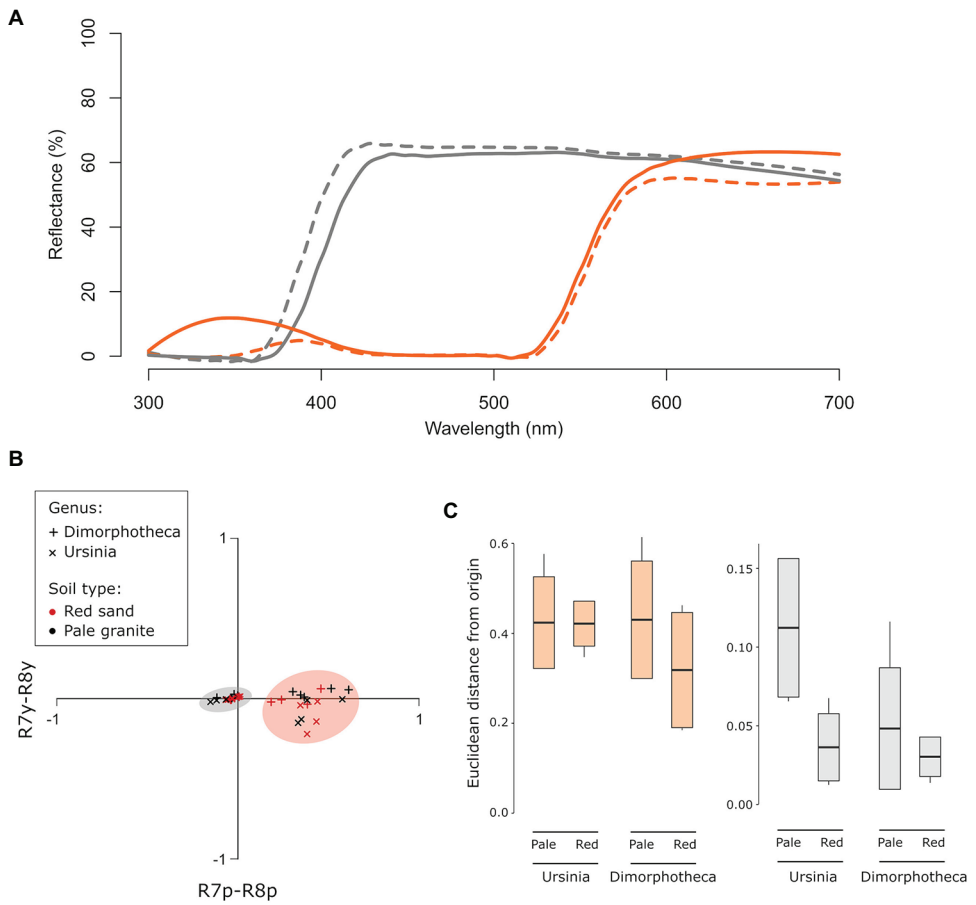


FIGURE 4 | Distinguishability and detectability of daisy flower color in fly vision. Orange flowers are indicated in orange and white flowers in gray throughout. **(A)** Reflectance spectra of ray florets of the four focal daisy species. Dotted lines indicate *Dimorphotheca* species, and solid lines show *Ursinia* species. **(B)** Flower colors modeled in the Troje (1993) fly color space using *Eristalis* receptor sensitivities with adaptation against the alternate soil backgrounds. The orange and gray circles indicate orange and white-flowered species, respectively. **(C)** Difference in detectability, quantified as Euclidean distances from the origin of fly color space, of the orange (*Dimorphotheca sinuata*, *Ursinia cakilefolia*) and white (*Dimorphotheca pluvialis*, *Ursinia speciosa*) species on the two soil backgrounds (pale and red soils). Species did not contrast significantly more strongly against their native soils (pale for orange flowers, red for white flowers) than foreign soils.

experimental studies of flies (e.g., Troje, 1993; Campbell et al., 2010; Lunau et al., 2018; Whitehead et al., 2019), the preference of *C. nigripes* for white flowers (80%) and *M. capensis* (91%) for orange flowers (**Figure 5**) is strong and unambiguous. While we cannot exclude the possibility that choices were influenced by traits other than flower color, the fact that color choices were consistent across two closely related plant species pairs from different genera argues against this possibility. Further, the hues involved are distinguishable under existing models of fly vision (**Figure 4**), and thus flower color presents a likely cue by which these plant species pairs could be discriminated by fly pollinators in our choice experiments. Given that the Troje (1993) categorical model of fly vision is unlikely to be universally applicable across the diverse visual systems of flies (Lunau, 2014; Hannah et al., 2019; Schnaitmann et al., 2020; van der Kooi et al., 2021), it is perhaps surprising that insights into color discrimination and detectability from our experimental and visual modeling approaches are so well aligned. Whitehead et al. (2019), for example, showed experimentally that *Prosoeca ganglbaueri* (Nemestrinidae) discriminates strongly between colors that are not distinguishable under the categorical fly vision model. Certainly, more research on the visual systems of important pollinating fly lineages, such as the Mariobezziinae, is required to further our understanding of their influence on floral trait evolution.

The strongly divergent color preferences demonstrated here could reflect innate differences in the visual systems of these flies, or alternatively, they could arise because the wild-caught flies used in our experiments were conditioned on the flower colors with which they are associated. As flower-visiting insects, including flies, are frequently able to learn chromatic cues (Dukas, 2008; Lunau et al., 2018), and mate-searching *M. capensis* males quickly learn to avoid female-mimicking floral ornaments (de Jager and Ellis, 2014a), we anticipated that our experimental results might reflect learning. However, we were unable to alter preferences through both short (1 h) and long (1 day)

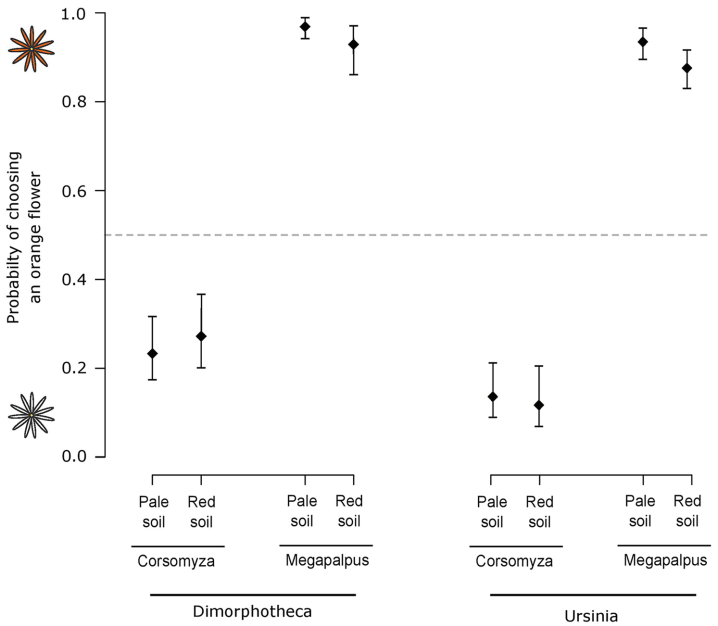


FIGURE 5 | The probability of pollinating flies (*Corsomyza nigripes* and *Megapalpus capensis*) choosing an orange flower over a white flower. Experiments tested fly color preferences using orange-white species pairs of *Dimorphotheca* (orange *Dimorphotheca sinuata* vs. white *Dimorphotheca pluvialis*) and *Ursinia* (orange *Ursinia cakilefolia* vs. white *Ursinia speciosa*) presented on both red and pale soils. *Megapalpus capensis* preferred orange flowers and *Corsomyza nigripes* preferred white flowers of both daisy genera, irrespective of the background-color.

term conditioning experiments, suggesting that the preferences we observe are spontaneous and not learned. While divergent spontaneous preferences are likely to reflect differences in the innate properties of the visual systems of *C. nigripes* and *M. capensis*, further experiments with naïve individuals, which are currently impossible as the life-histories of these flies are unknown, would be required to confirm this.

The background against which visual floral signals are viewed can influence both their detectability and discrimination (Bukovac et al., 2017). Because the parallel spatial gradients in flower color and pollinators highlighted here correspond to chromatically different soil backgrounds, we explicitly considered this neglected aspect of visual signaling in our experimental and modeling approaches. While visual modeling suggested that apparency of flowers did not differ across soil backgrounds (**Figure 4C**), choice experiments showed that white *Dimorphotheca* flowers are more detectable to *C. nigripes* on the red sand background, but that detectability of orange flowers did not differ across backgrounds for *M. capensis* (**Figure 6**). This apparent contradiction between modeling and experimental results may reflect the lack of information about photoreceptor sensitivities of the flies studied here, or general uncertainty in our understanding of fly color vision (van der Kooi et al., 2021). For example, recent work in *Drosophila* demonstrates the importance of inter-ommatidial opponency mechanisms in addition to the intra-ommatidial opponency pathways that underly existing fly color vision models (Heath et al., 2020). Alternatively, flies may use an achromatic processing mechanism (Lunau, 2014), to discriminate bright white flowers against the brighter granite and darker red soil backgrounds. Importantly, despite the influence of background coloration on detectability, strong spontaneous color preferences persist regardless of the background on which flowers were presented. Thus, while background coloration may influence signaling, and should be considered more explicitly in studies of flower color (Bukovac et al., 2017), it does not explain the landscape-scale color patterns described in this manuscript.

Fly Pollinator Mosaics

Our dataset is novel in that it estimates the "true" underlying pollinator gradient independent of the focal plant species and their phenotypic traits, something which is rarely done (e.g., Phillips et al., 2015). Because Mariobezziinae flies spend the vast majority of their time in flowers (AGE pers. obs.), by surveying all plant species within communities with which they associate (regardless of flower color), our survey approach provides a reliable estimate of fly densities that is independent of the focal plant species. The cause of these pollinator gradients is currently unclear and they could reflect abiotic (e.g., temperature) requirements (Hodkinson, 2005; Inouye et al., 2015) or host requirements of the parasitic larval stages of the bombyliid life-cycle (Yeates and Greathead, 1997). Further work is sorely needed to reveal the drivers of the distributions of these keystone fly pollinators that shape the pollinator climate across the greater cape floristic region.

In many respects, the strong pollinator gradients shown here are unexpected for plants such as daisies with a generalist pollination phenotype (i.e., open access flowers – Ollerton et al., 2007).

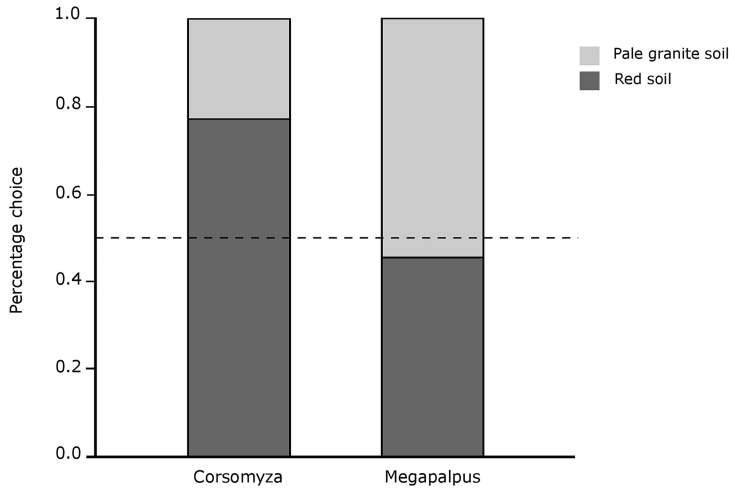


FIGURE 6 | Detectability of flower color on different background soils as quantified by initial choices of fly pollinators for flowers presented on different soil backgrounds. *Corsomyza nigripes* (usually found in white floral communities on red soils) chose white flowers (*Dimorphotheca pluvialis*) on a red soil background significantly more frequently that on pale soil, while *Megapalpus capensis* (usually found in orange floral communities on pale soils) visited orange flowers (*Dimorphotheca sinuata*) on both backgrounds with equal frequency. The dashed line indicates the expected choice ratio if there is no difference in detectability across backgrounds.

The gradients that underlie floral differentiation of generalist plants are usually thought to be more nuanced, comprising subtle quantitative differences in the relative availability of different pollinator species (or even functional types) in an assemblage of visitors that remains qualitatively largely unchanged across space (adaptive wandering – Thomson and Wilson, 2008; Gómez et al., 2014). In contrast to this model, the pollinator mosaic that we have quantified here is essentially qualitative, with near-complete turnover in the most abundant daisy visiting fly pollinator species (within the same pollinator functional group) across different habitats in the landscape. This is also true of the other common daisy visiting insect group in Namaqualand, the monkey beetles (Scarabaeidae, Rutelinae, Hoplinii). Monkey beetles also show strong species turnover, and evidence of variable color preferences and flower color associations, across habitat boundaries (Picker and Midgley, 1996; Colville et al., 2002; van Kleunen et al., 2007).

These strong pollinator gradients are also the likely driver of the surprisingly strong ecological specialization (i.e., interactions dominated by few pollinator species) of Namaqualand daisies described here and previously (Ellis and Johnson, 2009; Kemp et al., 2019), despite their generalist phenotypes (i.e., easily accessible pollen and nectar rewards). Because assemblages of potential daisy visitors in communities are dominated by a single abundant, highly-mobile fly species (densities were as high as 1.7 fly individuals m^{-2} for *M. capensis* and 3.7 m^{-2} for *C. nigripes*), these species dominate pollinator interactions, resulting in apparent (ecological) specialization of the plants. This effect is not contingent on floral traits that filter less effective pollinators, but results from the strong underlying pollinator density gradients. However, we cannot exclude the possibility that traits, such as pollen/nectar chemistry or disk floret tube lengths, are acting as filters, and thus contributing to pollination specialization, in Namaqualand daisies, something that is in fact suggested by the virtual absence of bees as pollinators (**Table 1**).

Ecological specialization sets up the potential for strong consistent divergent selection on floral traits, such as color in this case, in these generalist plants across gradients of turnover in dominant pollinators. Thus, these systems are potentially functioning more like the qualitative gradients underlying pollinator-shift models of divergence in phenotypically more specialized plants (Johnson, 2010). Our results suggest that pollinator-shift driven floral differentiation in space is not the sole provenance of pollination specialists, but is also expected in generalists whenever underlying pollinator gradients are strong and spatiotemporally stable enough to create a situation of ecological specialization despite phenotypic generalization.

Implications for Flower Color Differentiation

While our study convincingly demonstrates that a strong qualitative gradient of spatial turnover in dominant fly species underlies the spatial separation of orange and white-dominated daisy communities because of their divergent color preferences, the mechanistic link between these congruent spatial mosaics remains unclear. On the one hand, pollinator mediated divergent selection may have powered divergence of flower color across the gradient, or geographic variation in color preferences of dominant flies may act as an ecological filter, determining which flower colors are able to establish at different sites. Alternatively, it is also possible that fly color preferences have evolved across gradients of daisy flower color, or that fly communities are ecologically filtered by flower color. While further work is required to resolve this chicken or egg dilemma, some lines of evidence argue against the latter. First, the daisy

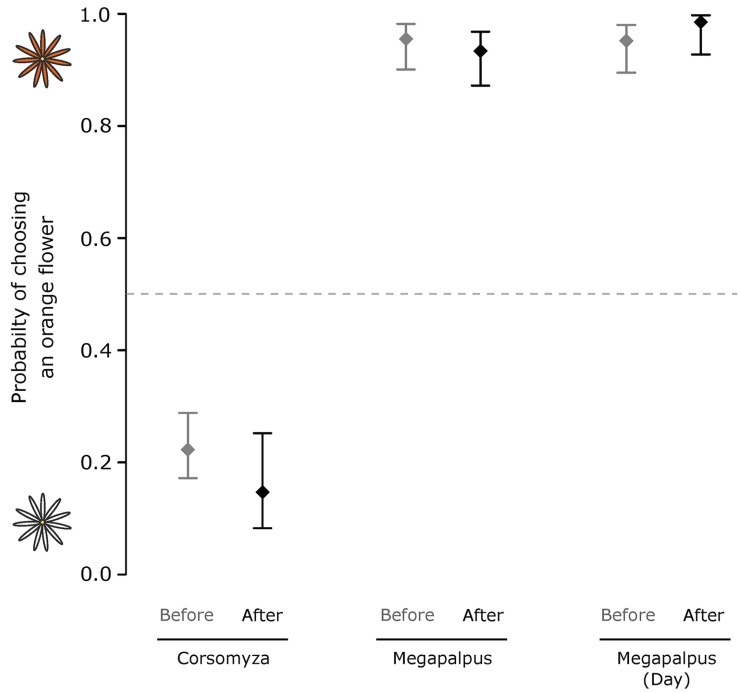


FIGURE 7 | The effect of color conditioning on the flower color choices of two dominant fly pollinator species. Choice experiments were used to characterize flower color choices before and after conditioning. In the first experiment *Corsomyza nigripes* and *Megapalpus capensis* flies were conditioned on flowers of their non-preferred color for 1 h, and in a second experiment, *Megapalpus capensis* flies were forced to feed on non-preferred white flowers for a day. Flower color preferences were not altered by conditioning.

clades involved are likely more recently evolved than the flies. The genus *Dimorphotheca* is estimated to have arisen 20.12 (8.94–27.88) mya (Barreda et al., 2015), and thus the sister species pair we studied is undoubtedly much younger, as the only taxonomically useful trait separating them is flower color (Norlindh, 1943). In contrast, the split between *Megapalpus* (a monospecific genus) and its sister genus, *Corsomyza*, is substantially older (26 my – de Jager and Ellis, 2017, 38 my – Li et al., 2020). This arguably makes it more likely that flower colors have diverged across a pre-existing geographic mosaic of fly species. Secondly, as *M. capensis* readily fed on white flowers when orange flowers were unavailable in the learning experiments, and is the primary pollinator of a guild of white-flowered pelargoniums in the southern part of its distribution (Struck, 1997), it is unlikely that flower color is limiting the distribution range of *M. capensis*. Regardless, the strong contemporary pollinator mosaic we demonstrate here is likely powering present-day flower color evolution and/or assembly. This is supported by widespread spatial mosaics of intraspecific color variation of annual, fly pollinated daisy species in Namaqualand (e.g., *G. diffusa* – Ellis and Johnson, 2009, *U. calenduliflora* – de Jager and Ellis, 2014b, *D. pinnatum* – Kemp et al., 2019).

Ours is not the first study to highlight the influence of gradients in the relative availability of fly pollinators on the spatial distribution of flower color. It has long been suggested that the dominance of yellow/white flower colors in high alpine communities reflects the relative importance of flies as pollinators because bees are at low density in these communities (Inouye and Pyke, 1988; Pickering and Stock, 2004; Bergamo et al., 2018). Similarly, a recent analysis of flower color on Macquarie Island, where bees are absent and flies dominate pollinator assemblages, showed an unusual prominence of cream-green flowers, that was attributed to filtering of plant colonists by fly pollinators (Shrestha et al., 2016). In both these examples, the assumption is that flies exert significant selection on flower color in generalist plants only when bees (which are assumed to be more effective pollinators because of strong flower constancy – Shrestha et al., 2016) are absent or of reduced importance. Interestingly, this assumption certainly holds in our study system, where bees are virtually absent as pollinators. This does not reflect their absence from the landscape though – bees are diverse and abundant in these Namaqualand communities (Kuhlmann, 2009) – but likely results because the daisies we studied produce very little nectar in comparison to the surrounding plant community, or are actively filtering bees through other means. Intriguingly, because bees have conserved visual systems and exhibit flexible responses to flower color (Chittka et al., 1999; van der Kooi et al., 2021), spatial gradients in the identity of available bee pollinators would not be expected to result in divergent selection on flower color or ecological sorting on the basis of flower color, as is implied by our results. Thus, the striking landscape-level divergence in dominant

flower colors that we have investigated here, and which has been demonstrated more widely by Kemp et al. (2019), might well be facilitated by the reliance of these annual daisies on fly pollinators.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AE and BA conceptualized the study. AE, JK, and BA collected the data. JK conducted the analyses with input from AE. AE and JK wrote the manuscript with input from BA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.617761/full#supplementary-material

REFERENCES

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly *Eristalis tenax* prefers different yellow colors for landing and proboscis extension. *J. Exp. Biol.* 221:jeb184788. doi: 10.1242/jeb.184788

Barreda, V. D., Palazzesi, L., Tellería, M. C., Olivero, E. B., Raine, J. I., and Forest, F. (2015). Asteraceous pollen in the cretaceous of Antarctica. *Proc. Natl. Acad. Sci.* 112, 10989–10994. doi: 10.1073/pnas.1423653112

Bergamo, P. J., Telles, F. J., Arnold, S. E. J., and de Brito, V. L. G. (2018). Flower color within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-5

Born, J., Linder, H. P., and Desmet, P. (2007). The greater cape floristic region. *J. Biogeogr.* 34, 147–162. doi: 10.1111/j.1365-2699.2006.01595.x

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017). Why background color matters to bees and flowers. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Campbell, D. R., Bischof, M., Load, J. M., and Robertson, A. W. (2010). Flower color influences insect visitation in alpine New Zealand. *Ecology* 91, 2638–2649. doi: 10.1890/09-0941.1

Chittka, L., Thomson, J., and Waser, N. (1999). Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86, 361–377. doi: 10.1007/s001140050636

Colville, J., Picker, M., and Cowling, R. (2002). Species turnover of monkey beetles (Scarabaeidae: Hopliini) along environmental and disturbance gradients in the Namaqualand region of the succulent Karoo, South Africa. *Biodivers. Conserv.* 11, 243–264. doi: 10.1023/A:1014520226989

Cooley, A. M., Carvallo, G., and Willis, J. H. (2008). Is floral diversification associated with pollinator divergence? Flower shape, flower color and pollinator preference in Chilean *Mimulus*. *Ann. Bot.* 101, 641–650. doi: 10.1093/aob/mcn014

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower color are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

de Jager, M. L., Dreyer, L. L., and Ellis, A. G. (2011). Do pollinators influence the assembly of flower colors within plant communities? *Oecologia* 166, 543–553. doi: 10.1007/s00442-010-1879-7

de Jager, M. L., and Ellis, A. G. (2012). Gender-specific pollinator preference for floral traits. *Funct. Ecol.* 26, 1197–1204. doi: 10.1111/j.1365-2435.2012.02028.x

de Jager, M. L., and Ellis, A. G. (2013). The influence of pollinator phylogeography and mate preference on floral divergence in a sexually deceptive daisy. *Evolution* 67, 1706–1714. doi: 10.1111/evo.12070

de Jager, M. L., and Ellis, A. G. (2014a). Costs of deception and learned resistance in deceptive interactions. *Proc. R. Soc. B Biol. Sci.* 281:20132681. doi: 10.1098/rspb.2013.2861

de Jager, M. L., and Ellis, A. G. (2014b). Floral polymorphism and the fitness implications of attracting pollinating and florivorous insects. *Ann. Bot.* 113, 213–222. doi: 10.1093/aob/mct189

de Jager, M. L., and Ellis, A. G. (2017). Evolutionary history of a keystone pollinator parallels the biome occupancy of angiosperms in the greater cape floristic region. *Mol. Phylogenet. Evol.* 107, 530–537. doi: 10.1016/j.ympev.2016.12.004

Desmet, P. G. (2007). Namaqualand – a brief overview of the physical and floristic environment. *J. Arid Environ.* 70, 570–587. doi: 10.1016/j.jaridenv.2006.11.019

de Waal, C., Anderson, B., and Ellis, A. G. (2015). Relative density and dispersion pattern of two southern African Asteraceae affect fecundity through heterospecific interference and mate availability, not pollinator visitation rate. *J. Ecol.* 103, 513–525. doi: 10.1111/1365-2745.12358

Dilley, J. D., Wilson, P., and Mesler, M. R. (2000). The radiation of *Calochortus*: generalist flowers moving through a mosaic of potential pollinators. *Oikos* 89, 209–222. doi: 10.1034/j.1600-0706.2000.890201.x

Dukas, R. (2008). Evolutionary biology of insect learning. *Annu. Rev. Entomol.* 53, 145–160. doi: 10.1146/annurev.ento.53.103106.093343

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Garcia, J. E., van der Kooi, C. J., and Wong, B. B. (2019). Color preferences of *Tetragonula carbonaria* Sm. Stingless bees for color morphs of the Australian native orchid *Caladenia carnea*. *J. Comp. Physiol. A.* 205, 347–361. doi: 10.1007/s00359-019-01346-0

Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.1093/beheco/ars019

Ellis, A. G., and Johnson, S. D. (2010). Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *Am. Nat.* 176, E143–E151. doi: 10.1086/656487

Ellis, A. G., and Johnson, S. D. (2012). Lack of floral constancy by bee fly pollinators: implications for ethological isolation in an African daisy. *Behav. Ecol.* 23, 729–734.

Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *Am. Nat.* 139, S125–S153. doi: 10.1086/285308

Fægri, K., and van der Pijl, L., (1966). *The principles of pollination ecology*. Oxford: Pergamon. 248.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* 91, 228–231. doi: 10.1007/s00114-004-0530-z

Giurfa, M., Nunez, J., Chittka, L., and Menzel, R. (1995). Color preferences of flower-naive honeybees. *J. Comp. Physiol. A.* 177, 247–259.

Goldblatt, P., and Manning, J. C. (2000). The long proboscid fly pollination system in southern Africa. *Ann. Missouri Bot. Gard.* 87, 146–170.

Gómez, J. M., Muñoz-Pajares, A. J., Abdelaziz, M., Lorite, J., and Perfectti, F. (2014). Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Ann. Bot.* 113, 237–249. doi: 10.1093/aob/mct186

Gómez, J. M., Perfectti, F., Bosch, J., and Camacho, J. P. M. (2009). A geographic selection mosaic in a generalized plant–pollinator–herbivore system. *Ecol. Monogr.* 79, 245–263. doi: 10.1890/08-0511.1

Halekoh, U., Højsgaard, S., and Yan, J. (2006). The R package geepack for generalized estimating equations. *J. Stat. Softw.* 15, 1–11. doi: 10.18637/jss.v015.i02

Hannah, L., Dyer, A. G., Garcia, J. E., Dorin, A., and Burd, M. (2019). Psychophysics of the hoverfly: categorical or continuous color discrimination? *Curr Zool* 65, 483–492. doi: 10.1093/cz/zoz008

Harder, L. D., and Johnson, S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytol.* 183, 530–545. doi: 10.1111/j.1469-8137.2009.02914.x

Heath, S. L., Christenson, M. P., Oriol, E., Saavedra-Weisenhaus, M., Kohn, J. R., and Behnia, R. (2020). Circuit mechanisms underlying chromatic encoding in *Drosophila* photoreceptors. *Curr. Biol.* 30, 264–275.e8. doi: 10.1016/j.cub.2019.11.075

Heinze, G., and Ploner, M. (2018). Logistf: Firth's bias-reduced logistic regression. R package version 1.23. Available at: https://CRAN.R-project.org/package=logistf (Accessed August 20, 2020).

Hodkinson, I. D. (2005). Terrestrial insects along elevation gradients: species and community responses to altitude. *Biol. Rev.* 80, 489–513. doi: 10.1017/S1464793105006767

Inouye, D. W., Larson, B. M. H., Ssymank, A., and Kevan, P. G. (2015). Flies and flowers III: ecology of foraging and pollination. *J. Pollinat. Ecol.* 16, 115–133. doi: 10.26786/1920-7603(2015)15

Inouye, D. W., and Pyke, G. H. (1988). Pollination biology in the snowy Mts. of Australia, with comparisons with montane Colorado, U.S.A. *Aust. J. Ecol.* 13, 191–210.

Johnson, S. D. (2006). "Pollinator-driven speciation in plants" in *Ecology and evolution of flowers*. eds. L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 295–310.

Johnson, S. D. (2010). The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philos. Trans. R. Soc. B* 365, 499–516. doi: 10.1098/rstb.2009.024

Johnson, S. D., and Midgley, J. J. (1997). Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *Am. J. Bot.* 84, 429–436.

Kemp, J. E., Bergh, N. G., Soares, M., and Ellis, A. G. (2019). Dominant pollinators drive non-random community assembly and shared flower color patterns in daisy communities. *Ann. Bot.* 123, 277–288. doi: 10.1093/aob/mcy126

Kemp, J. E., and Ellis, A. G. (2019). Cryptic petal coloration decreases floral apparency and herbivory in nocturnally closing daisies. *Funct. Ecol.* 33, 2130–2141. doi: 10.1111/1365-2435.13423

Koski, M. H. (2020). The role of sensory drive in floral evolution. *New Phytol.* 227, 1012–1024. doi: 10.1111/nph.16510

Koski, M. H., and Ashman, T. -L. (2015). Floral pigmentation patterns provide an example of Gloger's rule in plants. *Nat. Plants* 1:14007. doi: 10.1038/nplants.2014.7

Koski, M. H., and Galloway, L. F. (2020). Geographic variation in floral color and reflectance correlates with temperature and colonization history. *Front. Plant Sci.* 11:991. doi: 10.3389/fpls.2020.00991

Kuhlmann, M. (2009). Patterns of diversity, endemism and distribution of bees (Insecta: Hymenoptera: Anthophila) in southern Africa. *S. Afr. J. Bot.* 75, 726–738. doi: 10.1016/j.sajb.2009.06.016

Li, X., Teasdale, L. C., Bayless, K. M., Ellis, A. G., Wiegmann, B. M., Lamase, C. J. E., et al. (2020). "Phylogenomics reveals accelerated late cretaceous diversification of bee flies (Diptera: Bombyliidae)" in *Cladistics*.

Lunau, K. (2014). Visual ecology of flies with particular reference to color vision and color preferences. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Lunau, K., An, L., Donda, M., Hohmann, M., Sermon, L., and Stegmanns, V. (2018). Limitations of learning in the proboscis reflex of the flower visiting syrphid fly *Eristalis tenax*. *PLoS One* 13:e0194167. doi: 10.1371/journal.pone.0194167

Magee, A. R., Boatwright, J. S., and Mucina, L. (2014). Four new species of *Ursinia* (Asteraceae, anthemideae) from South Africa, with an updated key to the genus in Namaqualand. *Phytotaxa* 177, 137–145. doi: 10.11646/phytotaxa.177.3.1

Menzel, R., Gumbert, A., Kunze, J., Shmida, A., and Vorobyev, M. (1997). Pollinators' strategies in finding flowers. *Isr. J. Plant Sci.* 45, 141–156. doi: 10.1080/07929978.1997.10676680

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in andean solanaceae. *Evolution* 68, 2275–2286. doi: 10.1111/evo.12441

Newman, E., Anderson, B., and Johnson, S. D. (2012). Flower color adaptation in a mimetic orchid. *Proc. R. Soc. B Biol. Sci.* 279, 2309–2313. doi: 10.1098/rspb.2011.2375

Newman, E., Manning, J., and Anderson, B. (2015). Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution* 69, 2262–2275. doi: 10.1111/evo.12736

Norlindh, T. (1943). *Studies in the Calenduleae I: monograph of the genera Dimorphotheca, Castalis, Osteospermum, Gibbaria and Chrysanthemoides*. Lund: Gleerup.

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral color change in the eyes of pollinators: testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144–1155. doi: 10.1111/1365-2435.12420

Ollerton, J., Killick, A., Lamborn, E., Watts, S., and Whiston, M. (2007). Multiple meanings and modes: on the many ways to be a generalist flower. *Taxon* 56, 717–728. doi: 10.2307/25065855

Phillips, R. D., Bohman, B., Anthony, J. M., Krauss, S. L., Dixon, K. W., and Peakall, R. (2015). Mismatch in the distribution of floral ecotypes and pollinators: insights into the evolution of sexually deceptive orchids. *J. Evol. Biol.* 28, 601–612. doi: 10.1111/jeb.12593

Picker, M. D., and Midgley, J. J. (1996). Pollination by monkey beetles (Coleoptera: Scarabaeidae: Hopliini): flower and color preferences. *Afr. Entomol.* 4, 7–14. doi: 10.1111/j.1756-1051.2003.tb00384.x

Pickering, C. M., and Stock, M. (2004). Insect color preference compared to flower colors in the Australian Alps. *Nord. J. Bot.* 23, 217–223.

R Core Team (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Schnaitmann, C., Pagni, M., and Reiff, D. F. (2020). Color vision in insects: insights from *Drosophila*. *J. Comp. Physiol. A.* 206, 183–198. doi: 10.1007/s00359-019-01397-3

Shrestha, M., Burd, M., Garcia, J. E., Dorin, A., and Dyer, A. G. (2019). Color evolution within orchids depends on whether the pollinator is a bee or a fly. *Plant Biol.* 21, 745–752. doi: 10.1111/plb.12968

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., et al. (2016). Floral colors in a world without birds and bees: the plants of Macquarie Island. *Plant Biol.* 18, 842–850. doi: 10.1111/plb.12456

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Strauss, S., and Whittall, J. (2006). "Non-pollinator agents of selection on floral traits" in *Ecology and evolution of flowers*. eds. L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Struck, M. (1997). Floral divergence and convergence in the genus *Pelargonium* (Geraniaceae) in southern Africa: ecological and evolutionary considerations. *Plant Syst. Evol.* 208, 71–97.

Thomson, J. D., and Wilson, P. (2008). Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *Int. J. Plant Sci.* 169, 23–38. doi: 10.1086/523361

Troje, N. (1993). Spectral categories in the learning behaviour of blowflies. *Z. Naturforsch.* 48, 96–104.

Troscianko, J., and Stevens, M. (2015). Image calibration and analysis toolbox—a free software suite for objectively measuring reflectance, color and pattern. *Methods Ecol. Evol.* 6, 1320–1331. doi: 10.1111/2041-210X.12439

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66. doi: 10.1146/annurev-ento-061720-071644 (in press).

van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., and Midgley, J. J. (2014). Do pollinator distributions underlie the evolution of pollination ecotypes in the cape shrub Erica plukenetii? *Ann. Bot.* 113, 301–315. doi: 10.1093/aob/mct193

Van Kleunen, M., Nänni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle Marks and flower color on visitation by monkey beetles (Hopliini) in the greater cape floral region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256

Wang, H., Talavera, M., Min, Y., Flaven, E., and Imbert, E. (2016). Neutral processes contribute to patterns of spatial variation for flower color in the Mediterranean *Iris lutescens* (Iridaceae). *Ann. Bot.* 117, 995–1007. doi: 10.1093/aob/mcw036

Waterman, R. J., Bidartondo, M. I., Stofberg, J., Combs, J. K., Gebauer, G., Savolainen, V., et al. (2011). The effects of above- and belowground mutualisms on orchid speciation and coexistence. *Am. Nat.* 177, E54–E68. doi: 10.1086/657955

Whitehead, M. R., Gaskett, A. C., and Johnson, S. D. (2019). Floral community predicts pollinators' color preference: implications for Batesian floral mimicry. *Behav. Ecol.* 30, 213–222. doi: 10.1093/beheco/ary138

Yeates, D. K., and Greathead, D. (1997). The evolutionary pattern of host use in the Bombyliidae (Diptera): a diverse family of parasitoid flies. *Biol. J. Linn. Soc.* 60, 149–185.

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Why Variation in Flower Color May Help Reproductive Success in the Endangered Australian Orchid *Caladenia fulva*

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Caladenia fulva G.W. Carr (Tawny Spider-orchid) is a terrestrial Australian endangered orchid confined to contiguous reserves in open woodland in Victoria, Australia. Natural recruitment is poor and no confirmed pollinator has been observed in the last 30 years. Polymorphic variation in flower color complicates plans for artificial pollination, seed collection and *ex situ* propagation for augmentation or re-introduction. DNA sequencing showed that there was no distinction among color variants in the nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast trnT-trnF and matK regions. Also, authentic specimens of both *C. fulva* and *Caladenia reticulata* from the reserves clustered along with these variants, suggesting free interbreeding. Artificial cross-pollination *in situ* and assessment of seed viability further suggested that no fertility barriers existed among color variants. Natural fruit set was 15% of the population and was proportional to numbers of the different flower colors but varied with orchid patch within the population. Color modeling on spectral data suggested that a hymenopteran pollinator could discriminate visually among color variants. The similarity in fruiting success, however, suggests that flower color polymorphism may avoid pollinator habituation to specific non-rewarding flower colors. The retention of large brightly colored flowers suggests that *C. fulva* has maintained attractiveness to foraging insects rather than evolving to match a scarce unreliable hymenopteran sexual pollinator. These results suggest that *C. fulva* should be recognized as encompassing plants with these multiple flower colors, and artificial pollination should use all variants to conserve the biodiversity of the extant population.

Keywords: orchid, endangered, flower color, pollination, Hymenoptera, fruit set, DNA analysis

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INTRODUCTION

Conservation of endangered species is a high priority for members of the International Union for Conservation of Nature.[1] Such governments around the world are obligated to devise and fund strategies to prevent the extinction of endangered species, in order to conserve biodiversity. The Orchidaceae is one of the most threatened plant

[1]https://www.iucn.org/

families, as it has large numbers and proportions of endangered species worldwide (Wraith and Pickering, 2019).

Caladenia and Conservation

In temperate Australia, *Caladenia* species are widespread terrestrial orchids that produce a single leaf, may produce a single flower (occasionally 2), perennate by annual tubers and are active above-ground during cooler, wetter months, typically from late autumn through winter to spring (Jones, 2008). However, 18% of *Caladenia* species are threatened and protected under the Environment Protection Biodiversity and Conservation Act (EPBC) 1999 (Australian Government Department of Agriculture Water and the Environment, 2018). In the State of Victoria alone, 53% of the *Caladenia* species are listed as protected flora under the Victorian Flora and Fauna Guarantee Act (FFG) 1988 (Victorian Government Department of Environment, Land, Water and Planning, 2019). Most of these species are the very attractive spider-orchids in the subgenus Calonema (Hopper and Brown, 2004; Hopper, 2009; Clements et al., 2015). These are so named because of the long tapering tepals reminiscent of spider-legs.

One such threatened spider orchid is *Caladenia fulva* (Carr, 1991; **Figure 1**). The species is categorized as nationally endangered, and has had recovery plans under the EPBC and FFG for the last 18 years (Coates et al., 2002). Recovery plans for this, as for other endangered species, specify studying its biology and monitoring populations and their reproduction in the wild. Artificial pollination and seed collection are used to establish *ex situ* populations for conservation and use in possible augmentation or re-introduction *in situ* if natural pollination is low or absent (Coates et al., 2002). The major aim is to establish self-maintaining populations large enough to survive without human intervention.

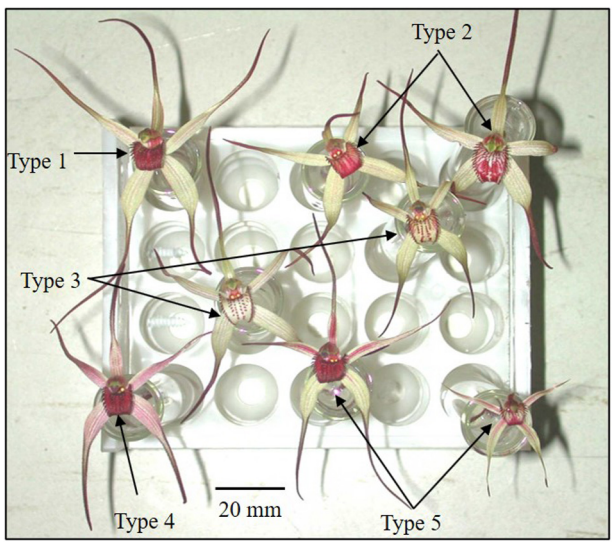


FIGURE 1 | Polymorphic flower color categories of *Caladenia* species from Deep Lead imaged with white light.

In *C. fulva*, no pollinator has been found despite baiting attempts (Colin Bower, personal communication) and plans for artificial pollination for seed production have been complicated by variation in flower color. *C. fulva* was described as having tawny (fawn)-colored tepals (three sepals and two petals) and a dark crimson labellum (Carr, 1991). The numerous color variants have various degrees of red blotching and streaks of red pigmentation in the tepals and labellum (**Figure 1**). The polymorphy in flower color has been speculated as being due to hybridization with other spider-orchids, especially *Caladenia reticulata* Fitzg. (Backhouse and Jeanes, 1995), but there has been no critical evidence to support this claim.

The uncertain status of the color variants, their unknown reproductive rates and the lack of an identified pollinator make it difficult to decide if all variants should be included or excluded in plans for artificial pollination. If the progeny are viable, either strategy could change the genetics of the populations and hence affect the conservation of the species. Molecular methods based on sequencing of nuclear and plastid genomes have been previously used in orchids to resolve taxonomic questions (Jones et al., 2001; Hopper, 2009; Breitkopf et al., 2015; Jin et al., 2017; Scaccabarozzi et al., 2018; Baguette et al., 2020) including in *Caladenia* (Swarts et al., 2014; Clements et al., 2015). Adopting these molecular methods is vital in the recovery plan to provide such information on population structure. It is also vital to test for the feasibility of cross-pollinating among color variants to find if fruits are formed and the seeds are viable.

Caladenia and Pollination

Most *Caladenia* spider-orchids investigated since the seminal paper by Stoutamire (1983) are pollinated by sexual deception of thynnine wasps (Hymenoptera-Thynnidae-Thynninae), e.g., Bohman et al. (2018). The male thynnine wasp is firstly attracted long-range (up to 10 m) by scents that imitate the pheromones of the wingless females (Bower, 1996; Schiestl, 2005; Peakall and Whitehead, 2014; Bohman et al., 2017; Phillips and Peakall, 2018; Wong et al., 2017). In such a case, color is therefore not the main or only attractant but links between flower color and odor have been demonstrated in other deceptive orchids (Dormont et al., 2014, 2019) and high contrast both within the flower and against the background is important (Gaskett et al., 2017; Phillips and Peakall, 2018).

Once such a pollinator lands on the central labellum of a sexually deceptive orchid, it attempts vigorously to copulate with the flower, thus acquiring the pollinia and depositing previously acquired pollen, before leaving to seek a mate elsewhere, typically outside the same orchid patch. For example, the thynnine wasp pollinators of the sexually deceptive *Drakaea glyptodon* and *Caladenia tentaculata* avoided multiple flowers within the same patch and in the latter case flew to a mean 17 m and up to almost 60 m away before depositing pollen (Peakall, 1990; Peakall and Beattie, 1996).

Bees and wasps (Hymenoptera) are the most common pollinators of sexually deceptive orchids (Bohman et al., 2018) and have the critical ability to learn the odor and appearance of

flowers after unsuccessful attempts to mate (Baguette et al., 2020). Flower color polymorphism may be advantageous by mitigating against the pollinator learning to avoid non-rewarding flowers (Wong and Schiestl, 2002; Wong et al., 2004; Schiestl, 2005; Jersáková et al., 2006; Gaskett and Herberstein, 2010; Gaskett, 2011; Dormont et al., 2014; Paulus, 2018; Dyer et al., 2019; Baguette et al., 2020). This has been termed negative frequency-dependent selection (Schiestl, 2005) and has the effect of keeping rarer flower types in the population. This is especially important in endangered orchids, in which populations are frequently small and pollination observations are limited (Tremblay et al., 2005; Phillips et al., 2009a, 2017).

Pollinators can be shared among orchids that overlap in range and flowering period (Joffard et al., 2019), but are shared less commonly among sexually deceptive species than among food-deceptive or rewarding (nectariferous) species (Phillips and Peakall, 2018; Joffard et al., 2019). However, most species investigated within the "*Caladenia reticulata/patersonii* complexes" (to which *C. fulva* belongs) shared pollination by one thynnine wasp – *Phymatothynnus nitidus* (Swarts et al., 2014). They thus formed one potentially interbreeding population in south-eastern Australia, over an area that overlaps that of *C. fulva*. At the other extreme, pollinator specialization has been found even within morphologically uniform populations of orchids and their pollinators (Bower and Brown, 2009; Phillips and Peakall, 2018), as well as within the *P. nitidus* complex (Phillips et al., 2009a).

Flower color variation in *C. fulva* could be significant in determining relative pollination and hence reproduction rate. Therefore, any selectivity in artificial pollination could affect conservation efforts for *C. fulva*. If one of the flower color phenotypes is more successful in pollination than the others, as in *Dactylorhiza sambucina* (Gigord et al., 2001), the population would be liable naturally to drift to a greater frequency of that phenotype or to reproductive isolation, as suggested in *Ophrys* evolution in Europe (Breitkopf et al., 2015). Counting the frequency of the flower color phenotypes in the *Caladenia* population and their success in fruiting would assist the recovery plan to monitor their relative frequency and likelihood of forming the next generation. Also testing the spectral qualities of flower color variants could assist the recovery plan by predicting which color variant(s) would be most attractive to hymenopteran pollinators.

Aims

The aims of this study were to assist the recovery plan for conservation of *C. fulva* by investigating (1) if DNA-based molecular grouping separated the flower color variants from one another and from "typical" *C. fulva* and *C. reticulata*, (2) if artificial pollination across flower color variants could produce viable seed, (3) if fruit formation varied among flower color variants after natural pollination, and (4) if a hymenopteran pollinator could discriminate amongst the different flower colors as seen by humans. Answers to these questions would benefit the recovery plan by clarifying if the flower color variants formed one interbreeding population and if artificial pollination was likely to change its structure. Finally, a rationale is proposed for the existence of polymorphic flower color in *C. fulva*.

MATERIALS AND METHODS

Site and Plants

The plants studied were growing at Deep Lead near Stawell in Western Victoria, Australia (latitude 37.0717°S, longitude 142.7908°E). The mean annual rainfall is 473.0 mm, the mean daily maximum temperature 20.6°C and the mean daily minimum temperature 8.5°C (Australian Government Bureau of Meteorology, 2020). The vegetation comprised of open woodland with *Eucalyptus leucoxylon* F.Muell. (yellow gum) as the dominant tree and a very sparse understory of the shrubs *Acacia pycnantha* Benth., *Acrotriche serrulata* R.Br., *Grevillea alpina* Lindl., *Micromyrtus ciliata* (Sm.) Druce, various grasses, and herbaceous perennials including 42 different orchid species (Carr et al., 1989). The area was assessed in the 2005 Ecological Vegetation Class (EVC) as a mixture of No. 61 Box-Ironbark Woodland and No. 48 Heathy Woodland with a strategic biodiversity value ranking of 100 (Victorian Government Department of Environment, Land, Water and Planning, 2020). The soil was a uniform red-yellow sandy gravel with a pH of 4.4 ($CaCl_2$) – 5.4 (water).

Caladenia fulva currently occurs only in part of two contiguous small flora and fauna reserves totaling 1120 ha in open woodland at Deep Lead, near Stawell, Victoria. Deep Lead was one of the richest alluvial fields in the Victorian goldfields and was highly disturbed during goldrushes in 1857–1878 and subsequent mining. *C. fulva* was first named in 1991 after a comprehensive study of the vegetation at Deep Lead (Carr et al., 1989; Carr, 1991). The description of *C. fulva* was based on one of the flower color variants at the site and the others were noted as being worthy of further investigation (Carr, 1991). The orchid is a summer-dormant herbaceous perennial that perennates by an annual succession of underground tubers that produce one green leaf and may produce a solitary flower each year. Its estimated population is 650 (Threatened Species Scientific Committee, 2016).

Caladenia fulva plants that grew in five patches 50–200 m apart were used for the study. Flowering plants were tagged and typed on 1–2 days in mid-flowering season in each of 2000 (81 plants), 2001 (49 plants), 2003 (70 plants), and 2004 (64 plants) (total of 264 plants). Each plant was only used once. The position of each orchid was tagged in the ground using a 2 mm stainless steel mini tent peg (Wiretainers, Brunswick East, Australia) to which was attached a uniquely numbered weatherproof pallid green plastic animal swivel tag (Stewart Farm Supplies).

Categorization of Color Variants

Each flower was categorized as one of five categories (**Table 1** and **Figure 1**) on the basis of tepal and labellum color. Category 1 corresponded to the type description of *C. fulva* (Carr, 1991; Geoff Carr, personal communication). Category 5 was closest to the type description of *C. reticulata* Fitz. A range of authentic flowers for each species is shown in a range of

TABLE 1 | Characteristics of polymorphic types of polymorphic *Caladenia* species at Deep Lead, and of *C. fulva* and *C. reticulata* (information on known species from Backhouse and Jeanes, 1995).

Species or type	Tepal color (to human eyes)	Labellum color (to human eyes)
Caladenia fulva	Yellow-green ± fine crimson stripes	Solid crimson
Category 1	Yellow-green ± fine crimson stripes	Solid crimson
Category 2	Yellow-green + crimson stripes or blotches	Crimson blotches or crimson with central white stripe
Category 3	Yellow-green or light yellow ± fine crimson stripes	Yellow-green or light yellow
Category 4	Pink-peach + crimson stripes	Solid crimson
Category 5	Yellow-green + thick central crimson stripe	Crimson, either solid or with blotches or central white stripe
Caladenia reticulata	Pale creamy yellow + basal red streaks and blotches	Solid crimson

See text for locations of illustrations of authentic species.

resources, including in the Flora of Victoria (Walsh and Entwisle, 1994, updated version online at https://vicflora.rbg.vic.gov.au/), Backhouse and Jeanes (1995), Jones (2008), Jeanes (2015), and the Atlas of Living Australia (2020).[2]

To record how closely mixed were the different categories of flower color, each tagged orchid in patches 1–4 was mapped in 2000–2001 using a theodolite relative to a tagged arbitrary point (0,0). A theodolite was used to record the distances between plants of different categories, as GPS accuracy was inadequate due to the close spacing (frequently < 5 cm) between individuals. Measurements recorded were bearing and distant from point (0,0), which was marked by a post in the ground. The mapping data were graphed manually to give (x,y) coordinates that were entered into a Microsoft Excel spreadsheet to produce a map of the tagged orchids that flowered in 2001.

Molecular Analysis

DNA was extracted from leaf tips of 31 plants that flowered in 2001 using a Qiagen DNeasy Plant Mini kit according to the manufacturer's instructions. Sample size was limited (4–80 mg, average of 36 mg) because the species is endangered. DNA was also extracted from five samples of each of authentic "*C. fulva*" and "*C. reticulata*" collected from Deep Lead (the latter generously gifted by I. and T. McCann of the Stawell Field Naturalists Club via Neville Walsh of the Royal Botanic Gardens Melbourne). All authentic specimens had identities confirmed by staff at the Royal Botanic Gardens before use.

Sequencing

One nuclear (ribosomal internal transcribed spacer – ITS) and several regions of the chloroplast genes (trnT-F, trnK) were sequenced as used previously for *Caladenia* (Hopper and Brown, 2004; Swarts et al., 2014; Clements et al., 2015). The ITS region was amplified using the universal primers ITS1 and ITS4 (White et al., 1990) as described previously (Reiter et al., 2018a). Four regions of the chloroplast genome were also sequenced using previously published primers and conditions: trnT-trnL, trnL intron, trnL-trnF (Taberlet et al., 1991) and the latter part of the matK region (here designated matK2) of the trnK intron (Sauquet et al., 2003; Steane et al., 2003). Each product was purified and sequenced as described previously (Reiter et al., 2018a). Products were electrophoresed at Micromon (Monash University).[3]

Phylogenetic trees for each type of sequence were constructed from ClustalW alignment (Thompson et al., 1994) using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with 500 bootstraps (Felsenstein, 1985) in MEGA7 (Kumar et al., 2016).[4] Sequences for *C. fulva* have been deposited in GenBank as Accession Numbers MT894435-MT894470 (ITS), MT914511-MT914554 (trnT-trnL), MT914555-MT914601 (trnL intron), MT950637-MT950682 (trnL-trnF), and MT966280-MT966314 (matK2). For outgroups, comparable sequences for species from other sections of *Caladenia* were obtained from GenBank using NCBI (National Center for Biotechnology Information).[5] Genetic distances (d) within and between categories were calculated using Jukes-Cantor analysis in MEGA7.

Sequences were also concatenated for all those samples (as in Swarts et al., 2014; Clements et al., 2015; Joffard et al., 2019) with all five sequences to give an overall assessment of the phylogenetic relationships among the phenotypes and authentic species. Concatenated sequences were analyzed by Maximum Likelihood analysis in MEGA7 and also by the MrBayes 3.2.6 plugin (Huelsenbeck and Ronquist, 2001) in Geneious Prime 2021.0.3[6] using the HKY85 substitution model, a burn-in of 100,000, a subsampling frequency of 200 and a chain length of 1,100,000 with Plant 5, Category 2 as the outgroup.

Inter-Simple Sequence Repeats

Inter-simple sequence repeats (ISSR) was used to assess diversity among polymorphic phenotypes in other regions of their DNA using microsatellite primers $(CAT)_5$, $(GTG)_5$, and $(GACA)_4$, similarly to the study by Swarts et al. (2014). Each 25 μL reaction contained: 12.5 μL Promega GoTaq Green Master Mix, 9.5 μL nuclease-free water, 1 μL microsatellite primer (25 μM) and 2 μL containing 5–20 ng of genomic DNA or sterile nuclease-free water. Thermocycling for each primer was as described by Elmeer et al. (2011) for $(CAT)_5$ and by Ryberg et al. (2011) for $(GTG)_5$ and $(GACA)_4$. PCR products were separated by electrophoresis and products recorded as before. The presence or absence of

[2]https://bie.ala.org.au

[3]www.monash.edu/researchinfrastructure/micromon

[4]http://www.megasoftware.net/mega.php

[5]http://www.ncbi.nlm.nih.gov/

[6]www.geneious.com

each amplicon was entered into a binary matrix in the statistical program Minitab Version 18[7] and individuals were grouped by similarity using multivariate analysis (principal components analysis and cluster analysis with complete linkage and squared Euclidean distance at $p = 0.05$). Genetic diversity was estimated by the number of expected alleles (N_e), observed (H_0), and expected (H_e) heterozygosity in GenAlEx6.51 (Peakall and Smouse, 2006, 2012). AMOVA was used to estimate the degree of population genetic variation (φ_{PT}) (equivalent of F_{ST} for binary data).

Pollination Studies

Natural Pollination

To investigate if the different individuals varied in success in fruiting varied with color morphotype or patch, 34 of the orchids tagged in 2000–2001 were observed for natural pollination, as judged by fruit set. Since these were the more common types not needed for the pollination scheme in 2001, there was the possibility of bias and a limited number of types. Therefore, natural pollination was also monitored separately in later years by typing and tagging individual flowering orchids and noting if they produced capsules. No data were collected in 2002 because all orchids were heavily grazed and did not flower due to the ongoing drought. A total of 76 orchids flowered and were tagged in 2003, when the drought broke, and 73 in 2004. Data on differences in flowering (2000, 2003, and 2004) and fruiting (2003 and 2004) with category and patch were organized into contingency tables and analyzed by means of Chi-square tests and other measures of association (Hartl and Clarke, 2007) against a null hypothesis of no difference among categories and patches using Minitab Version 18.

Artificial Pollination

All available flowering plants were artificially cross-pollinated between and within color variants in each of 2000 and 2001 years according to a previously determined matrix to incorporate all possible combinations between and within the five categories (**Table 2** and **Supplementary Table 1**): 48 plants on September 25, 2000 and 33 plants on September 12 and 24, 2001 (total of 81 plants). To avoid bias, each plant was only used in 1 year. The number of flowering orchids tagged was dependent on their frequency over both years and did not represent the five categories equally.

Only flowers that retained the parent pollinia on the column and did not have naturally deposited pollinia on the stigma were used for artificial pollination. For each orchid, the ripe pollinia were removed with a sterile toothpick and smeared on to the ripe stigma of another predetermined individual after its ripe pollinia had been removed. Each artificially pollinated orchid flower was covered with a small khaki cotton bag, closed at the bottom with an in-built cotton thread tie, and tied around a wooden skewer to support the capsule weight in order to prevent grazing, seed loss at capsule dehiscence and interference from the natural pollinator.

Capsules were collected when ripe (brown) on October 30, 2000 and on November 12 and 16, 2001. The stalk was cut below the bag and the capsules were transported back to the laboratory still enclosed inside the bags to avoid seed loss from split capsules. Once bags had been opened in the laboratory, capsules and seeds were stored in individually labeled paper envelopes over dry silica gel in a sealed container at 4°C.

Seeds were assayed for viability using the fluorescein diacetate (FDA) method of Pritchard (1985) but without surface-sterilization. Replicate samples of more than 50 seeds per capsule were tested between 11 and 43 days after collection. Seeds with the embryo completely stained (fluorescent) were considered viable. Minitab was used to analyze the effect of the pollen and ovule parent types on seed viability against a null hypothesis of no difference, using a non-parametric test (Kruskal–Wallis), since the data could not be normalized. Seeds from grazed, lost or moldy capsules were neither counted nor included in the statistical analysis.

TABLE 2 | Variation in flowering with category and patch in which they grew in unbiased tagged plants of *Caladenia* species at Deep Lead in 2000, 2003, and 2004.

Criterion	No. flowers				
	2000	2003	2004	Total	%
Category					
1	15	17	13	45	21.2 ± 0.5
2	26	18	12	56	26.4 ± 1.9
3	14	23	27	64	30.2 ± 1.8
4	19	8	4	31	14.6 ± 2.1
5	4	4	8	16	7.5 ± 0.6
Total	78	70	64	212	100.0
Patch					
1	28	15	20	63	29.7 ± 1.8
2	50	14	10	74	34.9 ± 6.0
3	0	7	3	10	4.7 ± 1.0
4	0	8	8	16	7.5 ± 1.3
5	0	26	23	49	23.1 ± 3.9
Total	78	70	64	212	100.0

UV Reflectance and Spectrophotometry

Hymenopterans are attracted by ultraviolet and yellow but also by high color contrast in the flowers and between the flowers and the background (Streinzer et al., 2010; Rakosy et al., 2012; Gaskett et al., 2017). The probability of accurate discrimination by a hymenopteran trichromat can be accurately modeled from the honeybee by determining the Euclidean distance between the respective loci in the color space (von Helversen, 1972a; Peitsch et al., 1992; Briscoe and Chittka, 2001; Gaskett and Herberstein, 2010; Garcia et al., 2017, 2020).

Consistent with permits, in 2001 eight flowers representing each flower category and some subtypes were cut at the stalk base, placed in test-tubes of water and immediately transported back to the laboratory on ice for spectral measurement. Hymenopteran pollinators have vision sensitive to UV radiation, and parts of flowers frequently have UV reflecting signals not normally visible to the human eye (Kevan et al., 2001). The reflectance spectra of sepals and labella were measured using a Varian

[7] minitab.com.au

DMS100 double beam UV-visible spectrophotometer fitted with a diffuse reflectance attachment at 10 nm intervals from 300 to 650 nm relative to a Varian pressed polytetrafluoroethylene powder standard.

To determine if flower color phenotypes were sufficiently spectrally variable as to be discriminated by the likely thynnine wasp pollinator, the spectral data were modeled in the Hexagon color space (Chittka, 1992), assuming typical illumination of a midday open sky equivalent to CIE D6500 (Judd et al., 1964; Wyszecki and Stiles, 1982) expressed as photon flux (Spaethe et al., 2001).We used the spectral sensitivity functions for the three different photoreceptors present in the honeybee (*Apis mellifera*) reported by Peitsch et al. (1992) assuming as adaptation background a 10% reflectance achromatic background (Dyer, 1998).

The probability of accurate discrimination of colors can be predicted from the geometric distance between respective flower colors in a given color space through a discrimination function that incorporates the psychophysics of how color differences are perceived by an animal (von Helversen, 1972a,b). Such a function has been determined for free flying honeybees from experimental data on color discrimination under absolute conditioning (Garcia et al., 2018, 2020), which we used with Euclidean distance in the Hexagon color space as the predictor (independent) variable. Specifically if the probability of discrimination exceeds 70% (von Helversen, 1972a,b) for respective flowers, or flower parts, such colors are above a discriminable threshold for a hymenopteran pollinator. Our analyses thus considered (i) if different flowers had colors that were above or below threshold and (ii) if the colors of the sepals and labella of the same plant were above or below threshold.

RESULTS

Sample Population – Tagged and Mapped Orchids

Eighty-one flowering orchids were first categorized and tagged in 2000, 49 in 2001, 70 in 2003, and 64 in 2004 (total of 264). Three orchids in 2000 could not be categorized; one had flowers with calli extending onto its tepals and the other two presented various abnormalities.

The mean number of categorized and tagged plants that flowered per year in 2000, 2003, and 2004 was 71 – 7 (excluding 2001 data because of bias). On average, there were 21% Category 1, 26% Category 2, 30% Category 3, 15% Category 4, and 8% Category 5 flowers (**Table 2**). The proportions of categories in tagged orchids were unequal in each year and in total ($\chi^2 = 36.1$, $p < 0.001$) with Categories 1–3 together comprising 77% of the total. The proportions were biased to less common types in 2001 ($\chi^2 = 5.6$, $p = 0.232$) because only those were still required for the artificial pollination matrix and so the 2001 data were excluded from all except artificial pollination analysis. The proportions of the categories did not differ significantly between 2000 and 2003 ($\chi^2 = 7.8$, $p = 0.098$) or 2003 and 2004 ($\chi^2 = 4.460$, $p = 0.347$) but did differ between 2000 and 2004 ($\chi^2 = 19.346$, $p = 0.001$) and overall ($\chi^2 = 22.0$, $p = 0.005$). The main difference was the greater proportion of Category 3 and lesser proportion of Category 4 between 2000 and 2003–2004.

The flowering orchids grew in two adjacent larger patches with smaller numbers of more scattered individuals in three smaller patches elsewhere on the site (**Figure 2**). There were no obvious differences in categories among patches ($\chi^2 = 7.032$, $p = 0.134$). Note that these were flowering orchids, not the entire population, as plants were only tagged on 1–2 days in mid-flowering season each year and there were also many plants without flowers.

Molecular Analysis

Sequencing

Of the 52 DNA samples tested, 48 produced a single ITS product, 49 a single trnT-trnL product, all 52 a single trnL intron product, 51 a single trnL-trnF product and 37 a single matK2 product and so were sequenced. Some sequences (5 ITS, 6 trnT-trnF, and 15 matK2) were of poor quality and were omitted from the final analysis. Alignment and concatenation of the remaining 33 sequences (which included at least three sequences from each of the five categories and authentic species) showed that sequence homologies varied from 96.3 to 97.9% except for two sequences (for plants 42 and 5). The phylogenetic tree formed one large clade containing all but these two sequences (**Figure 3**). Phenotypes and authentic species were mixed throughout rather than separating into categories or authentic species. This mixing also occurred when Maximum Likelihood was used to analyze sequences of each region separately (**Supplementary Figures 1**–**5**) or the concatenated sequences (**Supplementary Figure 6**). In MEGA7, calculated values for mean distance (d) were less for the nuclear ITS sequences (0.001–0.025) than for chloroplast sequences (0.000–0.114). For ITS sequences, mean diversities within and among categories (including *C. fulva* and *C. reticulata* samples) were all small (d = 0.000–0.001) and the coefficient of differentiation was 0.155 – 0.120. For chloroplast sequences, mean diversities were also small (0.001–0.006) and coefficients of differentiation were −0.006 – 0.012 to 0.082 – 0.036.

ISSR

The primers $(CAT)_5$, $(GTG)_5$, and $(GACA)_4$ all produced multiple bands. Cluster analysis resulted in five large clusters, each containing more than one phenotype and one or more authentic samples (**Figure 4**). Principal components analysis grouped all phenotypes together in one large cluster without separating by phenotype. The samples of authentic species were scattered among the category samples except for two samples of *C. fulva* and one of *C. tentaculata*. Heterozygosity values were N_e = 1.258 – 0.028 and unbiased H_e = 0.157 – 0.016 and Shannon's Information Index (I) was 0.242 – 0.022. AMOVA resulted in $\varphi_{PT} = 0.085$ ($p = 0.010$) for categorical samples alone, with 91% of variation within categories and only 9% among categories. Corresponding values including authentic *C. fulva* and *C. reticulata* were 0.130 (p = 0.001) with 87% of variation within categories and only 13% among categories.

Natural Pollination

A total of 134 plants was monitored for natural pollination (70 in 2003 and 64 in 2004) (**Table 3**). Only 21 (15.7%) produced a

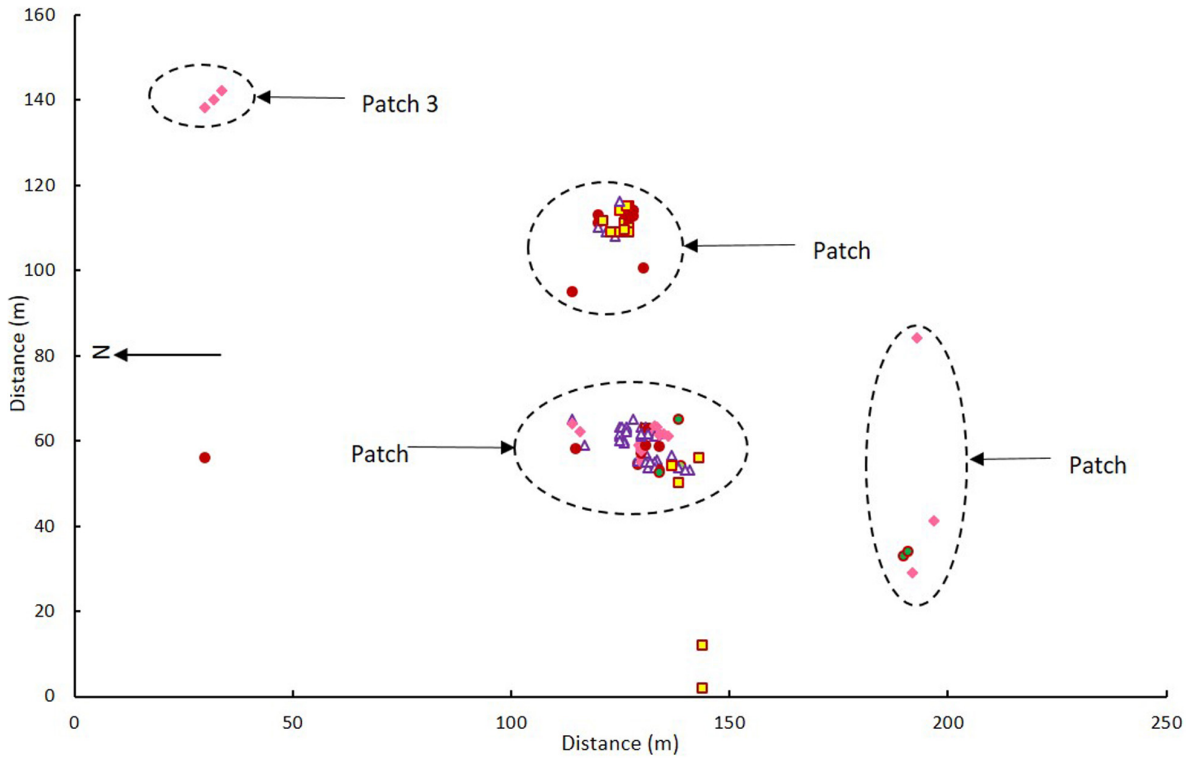


FIGURE 2 | Map of *Caladenia* categories at Deep Lead in patches 1–4 in 2000–2001. Key to flower types: Category 1 solid maroon circles, Category 2 white triangles with maroon borders, Category 3 yellow squares with maroon borders, Category 4 solid pink diamonds, Category 5 green circles with maroon borders.

capsule with viable seeds; this varied from 10/70 (14%) in 2003 to 11/64 (17%) in 2004.

Category of flower color did not affect success in fruiting. Success in fruiting following natural pollination ranged from 8% (Categories 4–5) to 13–20% (Categories 1–3) (**Table 3** and **Figure 5**). Plants in Categories 1–3 (those with 82% of the flowers) produced 91% of the fruits (Category 3 alone produced 43%). However, category itself had no significant effect on relative success in fruiting (χ^2 = 8.6, p = 0.072). There was also no significant deviation from expected numbers of fruit and flower numbers within Categories 1–3 (χ^2 = 0.358, p = 0.836). Alternative measures of association for data from all categories together also all had values close to zero (no association with category): Cramer's V-square = 0.008, Pearson's r = −0.035, Spearman σ = −0.030, Goodman–Kruskal's τ = 0.008, Kendall's τ-b = 0.027. By contrast, linear regression showed that the number of fruits was significantly affected by the number of flowers when data were arranged by category (fruit number = 0.2119 (flower number) – 1.4783, R^2 = 0.9562).

Patch strongly affected success in fruiting. Success in fruiting following natural pollination ranged from 1 to 10% for patches 3–5 to 25–31% for patches 1–2 (**Table 3** and **Figure 5**). Plants in patches 1–2 (with 44% of the flowers) produced 81% of the fruits on site. Patch number had a significant effect on flower number (χ^2 = 38.3, p < 0.001), fruit number (χ^2 = 17.8, p < 0.001) and % fruiting success (χ^2 = 38.3, p < 0.001). Linear regression showed no significant relationship of fruit number with flower number arranged by patch [fruit number = 0.0933 (flower number) + 1.6996, R^2 = 0.1125]. There was also other evidence of association of patch number with fruit number per patch, as values were all positive and deviated from zero: Cramer's V-square = 0.116, Pearson's r = 0.292, Spearman's σ = 0.314, Goodman–Kruskal's τ = 0.116, Kendall's τ -b = 0.284.

Artificial Pollination

Seventy of the 91 crosses (77%) in 2000–2001 resulted in the formation of capsules with seeds that could be tested for viability (**Table 4** and **Supplementary Table 1**). Of the remaining 21 artificially pollinated plants, seven did not form a capsule, eight were grazed, five had capsules that were moldy, and one plant could not be re-located. Capsule formation (not including grazed, lost or moldy capsules) was greater than 70% for all combinations, except for one (between Categories 3 and 5), in which only one capsule was produced from three crosses. Every combination except one (Category 3 with Category 5) produced a capsule with reciprocal crosses. Although crossing of Category 5 pollinia to Category 3 stigmas produced a capsule, the reciprocal cross (Category 3 pollinia to Category 5 stigmas) did not produce a capsule.

Seed viability by the FDA test varied from 0 to 97% with an average of 53% (**Table 4** and **Supplementary Table 1**). There was no significant difference in viability with either pollen parent category (Kruskal–Wallis, H = 3.59, p = 0.464) or ovular parent category (Kruskal–Wallis, H = 6.16, p = 0.188). Seed viability in

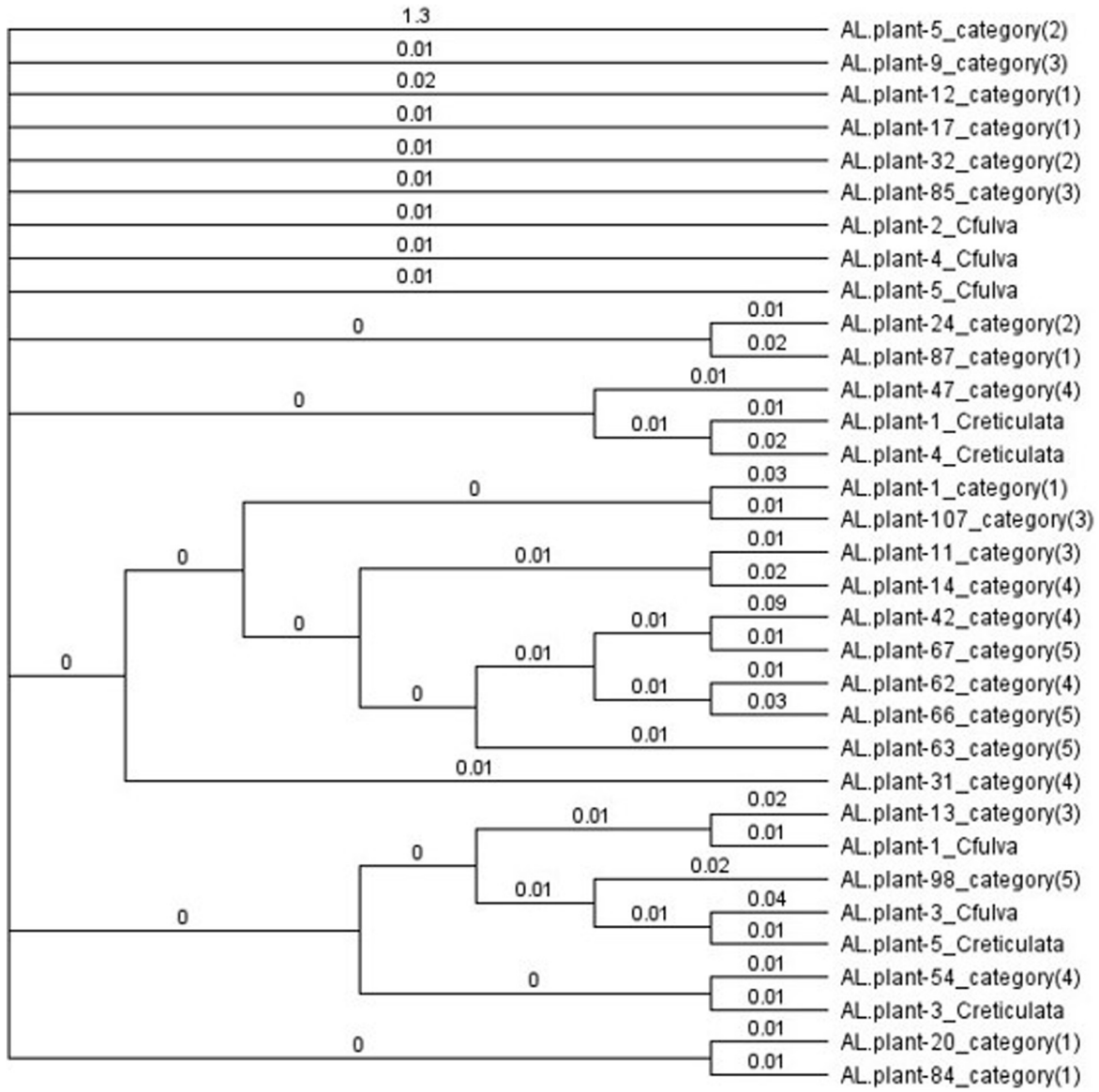


FIGURE 3 | Bayesian cladogram from concatenated ITS, trnT-F, and matK sequences, showing comparative relationships and intermixing of flower color categories and authentic samples of *Caladenia fulva* and *C. reticulata* collected in the same conservation reserve. Genetic distances derived from the posterior output are shown next to the branches.

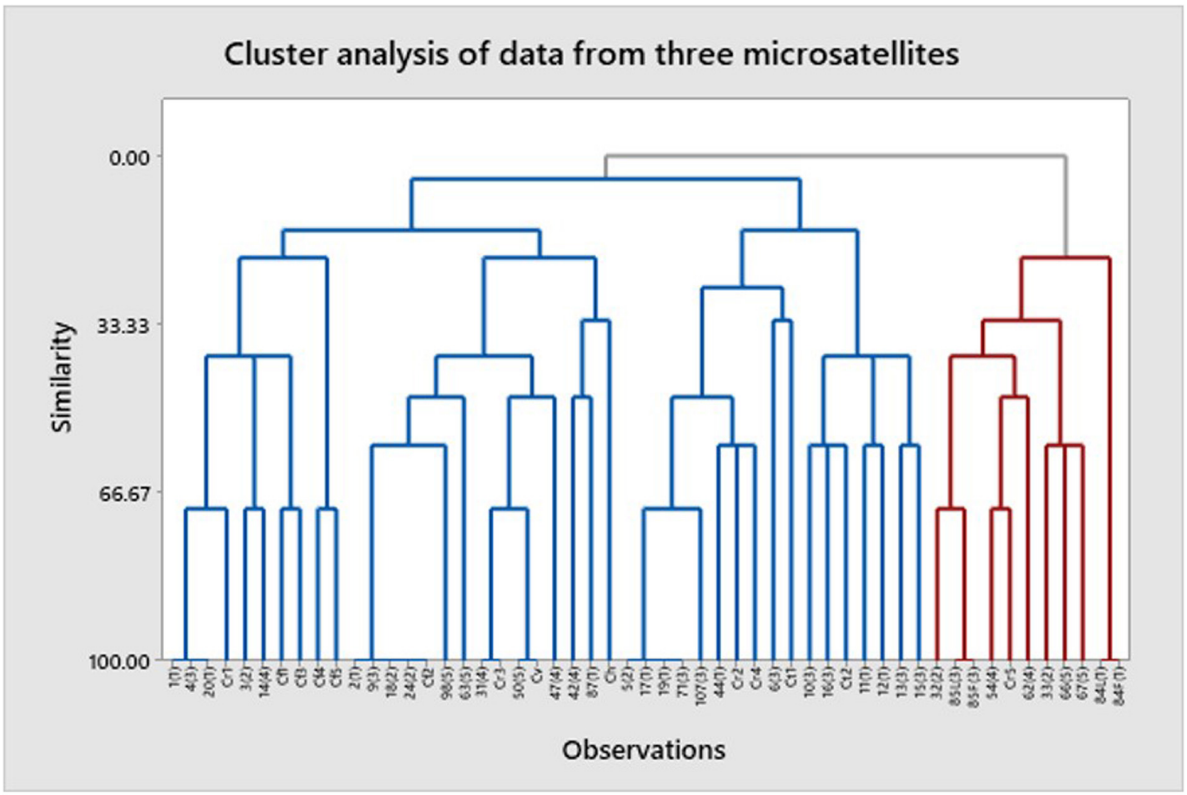
naturally pollinated plants in 2000 was 0–93% (mean 61 – 3%) in capsules from plants of Categories 1–4 (Category 5 plants had no capsules from naturally pollinated plants).

UV Reflectance and Spectrophotometry

Tepal reflectances showed an increase in reflection at about 400 nm (**Figure 6**), which is typical of hymenopteran-pollinated UV-absorbing white flowers around the world, including Australia (Kevan et al., 1996; Dyer et al., 2012; Bischoff et al., 2013).

To understand if the categorization of flower colors (**Table 1** and **Figure 1**) is relevant to the color visual system of hymenopteran trichromats it is important to consider two main scenarios including (i) whether different flowers had colors that were above or below the color threshold perceivable by a pollinator and (ii) if the sepals and labella of the same plant can be discriminated as from each other by a pollinator.

In scenario (i) and then separately comparing variance between the tepals of different plants, in 67% of cases these colors were above discrimination threshold, and there was also variance above threshold in 67% cases for the labella (**Table 5**). The evidence that flower color is variable in pollinator perception is confirmed by scenario (ii) where color within individual flowers comparing sepals and labella is above threshold in 67% of cases. Finally, comparing remaining possibilities (e.g., sepals versus labella comparisons of different flowers) then in 78%



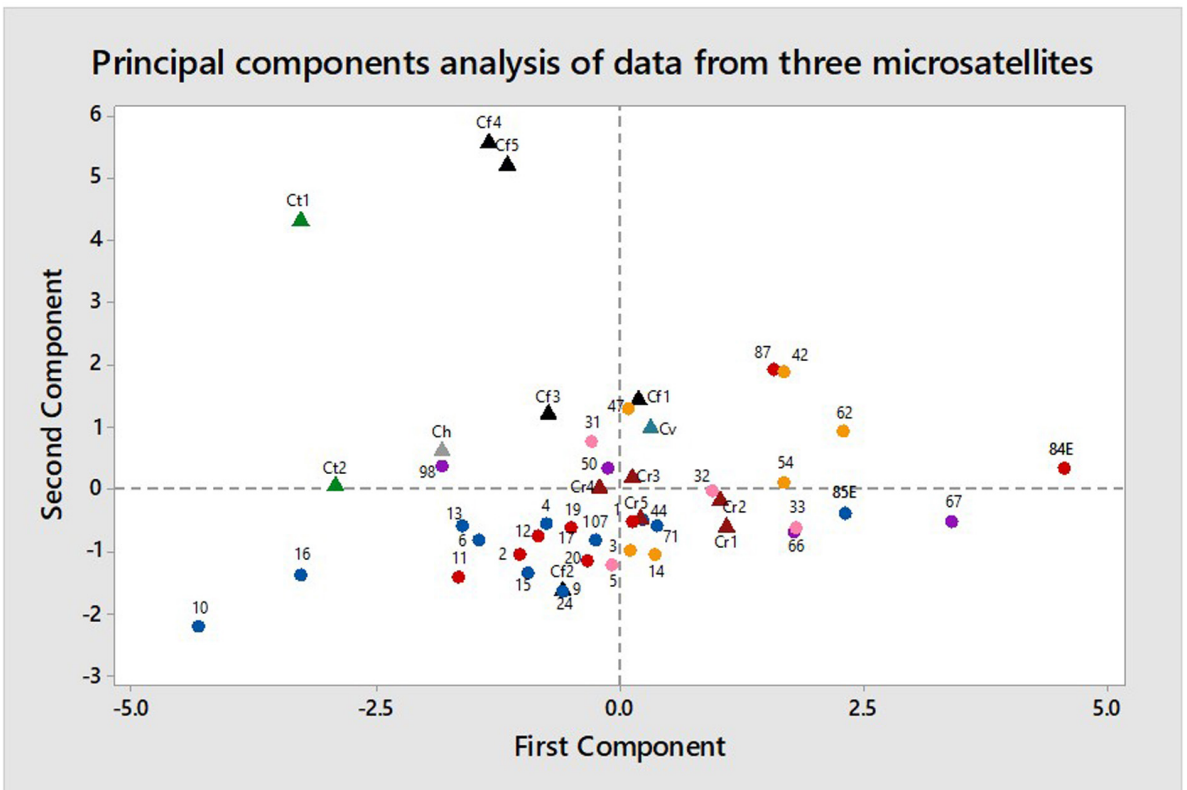


FIGURE 4 | Multivariate analysis of data from amplification of DNA of *Caladenia* species from Deep Lead and others by primers for three microsatellites: $(GACA)_4$, $(CAT)_5$, and $(GTG)_5$. Key to symbols: triangles, authentic species (black, *Caladenia fulva*; maroon, *C. reticulata*; gray, *Caladenia hastata*; teal, *Caladenia venusta*; green, *C tentaculata*); circles, specimens of polymorphic forms (red, Category 1; pink, Category 2; blue, Category 3; orange, Category 4; purple, Category 5).

TABLE 3 | Variation in flowering and natural pollination with category and patch in which they grew in *Caladenia* species at Deep Lead in 2003 and 2004.

Criterion	2003		2004		2003–2004		
	No. flowers	No. fruit	No. flowers	No. fruit	No. flowers	No. fruit	% success
Category							
1	17	3	13	1	30	4	13.3 ± 4.8
2	18	4	12	2	30	6	20.0 ± 4.6
3	23	2	27	7	50	9	18.0 ± 11.9
4	8	1	4	0	12	1	8.3 ± 2.4
5	4	0	8	1	12	1	8.3 ± 2.4
Total	70	10	64	11	134	21	15.7
Patch							
1	15	5	20	6	35	11	31.7 ± 1.7
2	14	3	10	3	24	6	25.7 ± 4.3
3	7	1	3	0	10	1	7.1 ± 7.1
4	8	0	8	1	16	1	6.3 ± 6.3
5	26	1	23	1	49	2	4.1 ± 0.3
Total	70	10	64	11	134	21	15.7

Overall 15.7% success (21/134).

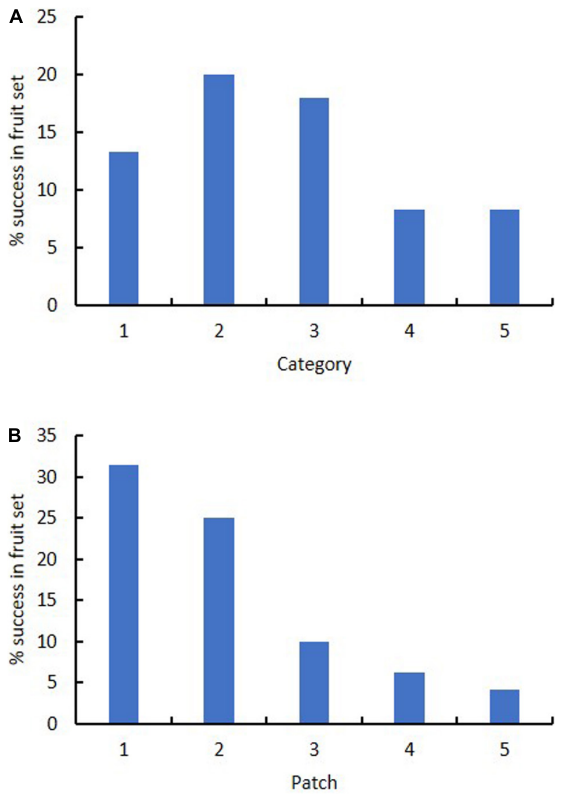


FIGURE 5 | Effect of **(A)** flower color category (1–5) and **(B)** patch (1–5) on percentage success in fruit set in *Caladenia* species at Deep Lead in 2003–2004.

of cases flowers had coloration above discrimination threshold (**Table 6**). These results show that the color variation used to classify flowers by human observers is often perceivable by hymenopteran pollinators.

DISCUSSION

The overarching aim of this study was to assist the recovery plan for conservation of *C. fulva* by deciding if all flower color variants (categories) should be included or excluded in plans for artificial pollination and eventual use of *ex situ* plants raised from such seeds for augmentation of the wild population or re-introduction.

The evidence presented here suggests that all flower color categories should be included in recovery actions for *C. fulva*. Firstly, phylogenetic trees did not separate categories or putative parental species and all measures of diversity were small. Secondly, there were no differences in natural seed set among categories of flower color and so no category was disadvantaged in pollination. Thirdly, there were no post-zygotic barriers to artificial cross-pollination or differences in viability in the seeds produced. Lastly, modeling pollinator color vision both within and between flowers suggested that a hymenopteran pollinator should frequently be able to discriminate amongst categories of flower color; the significance of this is discussed below.

Conservation

All molecular evidence suggests that all categories and the authentic samples of *C. fulva* at the site belong to one potentially interbreeding population that consists of one molecular operational taxonomic unit (MOTU) (Floyd et al., 2002). Since all patches contained all phenotypes of *C. fulva*, the origin of the flower color variation is likely to be genetic rather than environmental. The scattering among the clades of sequences of individuals collected as *C. reticulata* in the same reserves suggests that they too are not reproductively isolated from *C. fulva*. The descriptions of *C. fulva* and C. *reticulata* overlap, as do their ranges (Jeanes, 2015). Carr (1991) thought that *Caladenia audasii* might be a "derived form of *C. fulva*" and sequencing of the sole plant suspected to be "*C. audasii*" at the site would be worthwhile. Swarts et al. (2014) found that some

TABLE 4 | Capsule formation (% and number excluding moldy ones) and seed viability (%) of *Caladenia* categories from *in situ* artificial pollination experiments.

Criteria	Capsule formation (%) No. capsules formed (n)					Seed viability (%) (mean ± SE)				
Category	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Category 1	100	71	100	100	100	60 ± 1	53 ± 17	82 ± 3	60 ± 3	54 ± 7
	n = 7	*n* = 7	*n* = 6	*n* = 7	*n* = 2					
Category 2		100	100	100	100		60 ± 13	51 ± 14	55 ± 10	45 ± 15
		n = 6	*n* = 8	*n* = 6	*n* = 7					
Category 3			86	83	33			45 ± 14	30 ± 14	20 ± 20
			n = 7	*n* = 6	*n* = 3					
Category 4				100	100				19 ± 19	79 ± 15
				n = 2	*n* = 2					
Category 5					100					89
					n = 1					

TABLE 5 | Color and green contrast values for the measured sepals (*n* = 3) and labella (*n* = 4) of *Caladenia* species from Deep Lead.

Stimulus	Category	Green contrast	Color contrast
Orchid 1 sepal	1	0.267	0.345
Orchid 1 labellum	1	0.087	0.097
Orchid 2 labellum	2	0.091	0.133
Orchid 5 sepal	3	0.285	0.353
Orchid 5 labellum	3	0.188	0.294
Orchid 6 sepal	4	0.130	0.225
Orchid 6 labellum	4	0.163	0.033

other species in the *C. reticulata/Caladenia patersonii* complexes in this region of south-eastern Victoria were indistinguishable by similar molecular analyses and shared the same thynnine wasp pollinator. Further investigations similar to those described here using more discriminatory sequences or next-generation sequencing may elucidate the relationships among spider-orchids in the *C. patersonii/C. reticulata* complexes.

The high fruit set (77%) and seed viability (53%) from artificial pollination suggest that there are no post-zygotic barriers to cross-pollination among flower color categories at the site, as is common in orchids (Scopece et al., 2014; Baguette et al., 2020). The average seed viability was similar to that found in other orchids (Pritchard, 1985; Huynh, 1999; Sharma et al., 2000). Artificial pollination normally results in consistently greater fruit set than in natural pollination (Alexandersson and Agren, 1996; Primack and Stacy, 1998). It would be ideal to perform classical Mendelian studies of the flower colors of the progeny but there are currently considerable technical difficulties in raising all progeny to maturity (Wright et al., 2009). Repetitive use of plants to increase population size for conservation purposes must also take into account the cost of reproduction on the individual plant (Snow and Whigham, 1989; Primack and Hall, 1990; Alexandersson and Agren, 1996; Primack and Stacy, 1998; Tull, 1998; Coates et al., 2006; Coates and Duncan, 2009).

Natural Pollination

Fruit set occurred in only 16% of flowers and thus appears low, but is consistent with fruit set data reported for sexually pollinated rather than food-rewarding or food-deceptive species (Gregg, 1989; Tremblay et al., 2005; Jersáková et al., 2006; Vandewoestijne et al., 2009; Scopece et al., 2010; Swarts et al., 2014; Gaskett, 2011; Stejskal et al., 2015; Baguette et al., 2020), including *Caladenia* (Phillips et al., 2009b). This figure is common among natural populations of orchids with a variety of pollination strategies (Nilsson, 1979; Gill, 1989; Waite et al., 1991; Tull, 1998; Waite and Farrell, 1998; Willems and Melser, 1998) and is unlikely to limit recruitment (Tull, 1998).

Patches 1 and 2 together contributed 81% of the seed on site from only 44% of flowers, as noted in two other orchids overseas (Zimmerman and Aide, 1989; Tull, 1998). These patches were the largest on the site, by contrast with the negative trend in fruit set with flower number in European orchids (Tull, 1998; Baguette et al., 2020). Perhaps only large clusters of *C. fulva* produce enough odor to attract pollinators. Baiting attempts may thus need to include larger numbers of potted plants, as for *Caladenia colorata* (Reiter et al., 2018b). Also patches 1 and 2 are on the top of a small rise and are less shaded and therefore sunnier than other patches. Thynnine wasps are more common in sunny warm spring days (Peakall, 1990; Bower, 1996). So long as the flower color phenotypes in patches 1 and 2 continue to resemble those in the population, this bias is unlikely to change the relative proportions of flower color phenotypes in future.

The spectral profiles of the color signals displayed by *C. fulva* in a model of hymenopteran color vision are consistent with pollination by a hymenopteran insect (Chittka, 1992; Dyer et al., 2012; Garcia et al., 2017, 2018, 2020) and observed color and shape variation can be explained as an adaptation to facilitate pollination by hymenopteran insects in this orchid species. This is critical in understanding why *C. fulva* retains a variety of flower colors and patterns, when the plants with the flowers most attractive to a pollinator should be the most successful in reproduction and the population should gradually drift genetically to favor that type (Schiestl and Johnson, 2013). Two factors may be important here: (1) the ability of hymenopteran pollinators to learn from experience and (2) the likely availability of potential hymenopteran pollinators.

Firstly, hymenopterans such as bees and wasps are capable of learning colors and patterns of rewarding and non-rewarding flowers (e.g., Howard et al., 2019). A rewarding orchid flower that

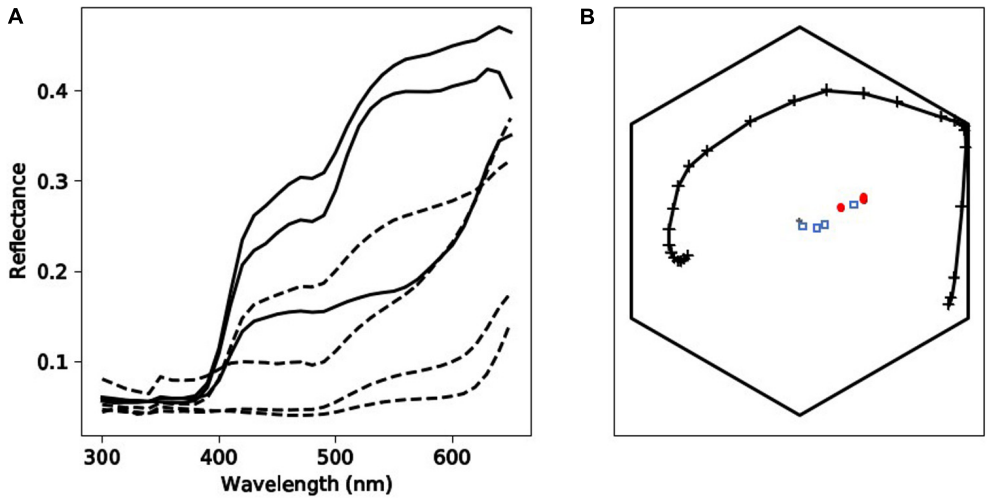


FIGURE 6 | Spectral qualities of *Caladenia* flowers from Deep Lead. Panel **(A)** depicts spectral profiles of tepal (solid black lines) and labellum (dashed black lines) regions of flowers from four different categories (1–4) of the orchid *Caladenia fulva*. Panel **(B)** shows the position of loci corresponding to the samples of *C. fulva* in the hexagon color model. In this panel, marker types identify the different flower regions: tepals (red circle markers) or labella (blue square markers). The spectral loci for *Apis mellifera* as a model of a trichromatic hymenopteran pollinator in the Hexagon color space is indicated by the solid, black line with cross markers.

TABLE 6 | Lower triangular matrix containing Euclidean distances between each possible pair of orchid samples (black normal font) and their corresponding probability of discrimination (blue bold italic font) as predicted by the color discrimination function for *Apis mellifera* when considering absolute conditioning.

	Category	O1 sep.	O1 lab.	O6 sep.	O6 lab.	O5 sep.	O5 lab.	O2 lab.
O1 sep.	1	0						
	1	*0.5*						
O1 lab.	1	0.274	0					
	1	*0.842*	*0.5*					
O6 sep.	3	0.130	0.157	0				
	3	***0.840***	***0.842***	*0.5*				
O6 lab.	3	0.333	0.066	0.208	0			
	3	***0.842***	*0.505*	***0.842***	*0.5*			
O5 sep	4	0.011	0.284	0.137	0.341	0		
	4	*0.5*	*0.842*	*0.841*	*0.842*	*0.5*		
O5 lab.	4	0.056	0.222	0.074	0.278	0.063	0	
	4	*0.5*	***0.842***	*0.570*	*0.842*	*0.5*	*0.5*	
O2 lab.	2	0.238	0.036	0.121	0.097	0.248	0.185	0
	2	***0.842***	*0.5*	***0.837***	*0.781*	***0.842***	***0.842***	*0.5*

A probability of discrimination equal to 0.5 indicates that choices are done at random, i.e., a bee is unable to discriminate a difference in color. Stimuli names are abbreviated from those in ***Table 3****: orchid (O), sepal (sep.), and labellum (lab.).*

delivers food such as nectar will be remembered and visited in future whereas a non-rewarding one will be avoided in future. Therefore, there is potentially an advantage for non-rewarding orchids in having a variety of flower colors and patterns that encourage multiple hymenopteran visits to flowers with slightly different colors or scents. The lack of reward with more common flower colors means that rarer flower colors are actively sought after and so the rarer categories persist in the population in proportion to their total flower numbers. This has been termed negative frequency-dependent selection (Smithson and Macnair, 1997). The learning abilities of hymenopterans are vital in this strategy (Garcia et al., 2020), as pollinators without such memories are indiscriminate in the search for the next flower.

Such signal variability has previously been shown to promote fitness benefits for orchids with non-rewarding flowers (Wong and Schiestl, 2002; Wong et al., 2004; Gaskett, 2011; Dyer et al., 2019; Jiménez-López et al., 2020). For example, sexually deceptive orchid *Ophrys* species display different spatial patterns in conspecific flowers (Stejskal et al., 2015; Paulus, 2019). This avoids pollinators habituating to non-rewarding signals and so increases the probability of multiple flower visits by an individual pollinator (Schiestl, 2005; Jersáková et al., 2006; Delle-Vedove et al., 2017; Dyer et al., 2019). Field experiments suggest that bees prefer visiting similar but discriminable flowers rather than distinctly different colors, a perceptual magnet-type effect that may benefit rarer colored flowers in

non-rewarding flower species (Peter and Johnson, 2008; Dyer and Murphy, 2009). Thus species with non-rewarding flowers appear to benefit from having dissimilar colors (Gaskett, 2011; Rakosy et al., 2012) and scents (Delle-Vedove et al., 2017) that potentially confuse the decision-making of pollinators and encourage outcrossing in the orchid (Streinzer et al., 2010; Paulus, 2019). Our observations from *C. fulva* using several lines of inquiry are consistent with this model, and explain the presence of the color variations previously reported for human observers.

Secondly, the availability of a reliable pollinator would be expected to drive plants with a variety of flower colors to evolve in the direction of the flower color most attractive to a reliable pollinator. Different flower colors may appeal to different potential pollinators in the plants' habitat and so variety in flower color (purple or yellow) may result in pollinator specialization, as in the orchid *D. sambucina* (Gigord et al., 2001). It may lead even further, into speciation, as in the sexually deceptive orchid genus *Ophrys* in Europe (Baguette et al., 2020) and *Drakaea* in Australia (Gaskett et al., 2017). In *Ophrys*, directional selection in favor of attractiveness to specific hymenopteran pollinators is thought to have led to the scent and appearance of the labellum in the small flowers being gradually modified to resemble a female bee that is attractive to a flying male bee seeking a mate, e.g., *Drakaea* (Gaskett et al., 2017), *Ophrys* (Baguette et al., 2020). This strategy works well when potential pollinators are abundant in highly conserved habitats.

However, this strategy works poorly when potential hymenopteran pollinators are sparse and thus unreliable in highly disturbed habitats where orchids and their pollinators are few. In this case, as in *C. fulva* in the Victorian goldfields, there is insufficient selection pressure toward modification of the labellum or tepals to resemble a female hymenopteran. Food-rewarding orchids are believed to precede both food-deceptive and sexually deceptive species (Weston et al., 2014). Therefore, there are advantages in maintaining the attractive bright colors of ancestral rewarding species and possibly obtaining pollination by suitably sized foraging hymenopterans or other flying insects. For example, red and pink colors increased pollinator attraction in *Ophrys heldreichii* (Streinzer et al., 2009; Spaethe et al., 2010). Also, the early stages in biochemical pathways for color and scent molecules are shared and so changes in color may be linked to differences in scent (Zuker et al., 2002; Knudsen et al., 2006; Majetic et al., 2007; Dormont et al., 2014, 2019).

A further point is that few alternative food-rewarding plants are available to foraging invertebrates in the sparse dry undergrowth. Even small quantities of nectar may constitute an important food source. Evidence that many orchids are truly rewardless is scarce and requires further research (Shrestha et al., 2020). The observation of a *Diamma* species licking the bases of the calli on the labellum (Kuiter, 2016) suggests that nectar may be produced directly on to the labellum, as in *Caladenia concolor* (Reiter et al., 2018b, 2019) and *C. nobilis* (Phillips et al., 2020). Given that encounters with foraging flying insects may be simply by chance, there would be advantages to maintaining a variety of attractive colors. Thus in both cases there are potential advantages for *C. fulva* in maintaining flower color diversity.

Clearly identification of the pollinator(s), analysis of the scents of the different color categories and examination of nectar secretion are needed to resolve these questions. Some orchids are pollinated by bees during the day as well as by moths at night (Delle-Vedove et al., 2017) and so diurnal observations should be included in attempts to find pollinators. Schiestl and Schluter (2009) have also suggested that, following scent, size is more important in pollinator attraction than flower color. *C. fulva* flowers are described as varying greatly in tepal size (50–80 mm long × 5–8 mm wide) (Jeanes, 2015). Extreme differences in the size of flowers were noted on site only in Category 5 but measuring flowers and their relative success in fruiting may reveal critical dimensions important for pollination.

The yellow-red flower color polymorphism in *C. fulva* is closest to type D of Dormont et al. (2019). As all plants produce deep red color in some parts of the flower, logically all the plants in the population at the site must possess all the required enzymes of the flavonoid synthesis pathway. This pathway is well characterized and is controlled by regulatory genes, resulting in white, yellow, deep red, and purple pigments, depending on the activities of different regulatory genes (Mol et al., 1998; Martens et al., 2003; Narbona et al., 2018; Jiménez-López et al., 2020). Baguette et al. (2020) recently suggested such epigenetic variation to be key to rapid speciation in *Ophrys* species and pointed out that this would explain the lack of genetic variation found by DNA sequencing despite large differences in odor compounds and color patterns among *Ophrys* species, which is also true for *Caladenia* species (Swarts et al., 2014). Further investigation could test for differences in the flavonoid enzymes and their regulatory enzymes among flower color categories.

Conservation Recommendations

Caladenia fulva is typical of polymorphic endangered orchids in that it has a small spatial range and its taxonomic limits are unclear, as in several such orchids worldwide, notably *Ophrys* species in Europe (Vereecken et al., 2010; Schatz et al., 2014; Joffard et al., 2019; Baguette et al., 2020). The results of this study suggest that *C. fulva* should be regarded as one interbreeding species that is polymorphic in flower color (Huxley, 1955; Narbona et al., 2018; Jiménez-López et al., 2020). Flower color polymorphism may be important in enticing a variety of hymenopterans to visit multiple flowers of *C. fulva* and thus enhance natural pollination and outcrossing even when pollinators are scarce.

This study has also resulted in the following recommendations, which are applicable to other polymorphic endangered orchids in which key facts are unknown. For the purpose of establishing an *ex situ* collection for conservation and possible augmentation or re-introduction, the safest option would be only to use seeds from naturally pollinated fruit of different flower colors. If artificially pollinated, all flower colors should be included (in the proportions found on site) to minimize interference with any natural genetic drift in flower color. Variation in numbers and proportions of flower color categories and their fruit set should be monitored regularly to detect any possible natural drift, e.g., increase in frequency of

Category 3. Sustained effort should be devoted to finding the natural pollinator(s) and essential requirements, including adequate abundance of food species for the pollinator(s) in the very sparse understory, in order to conserve rather than preserve *C. fulva*.

DATA AVAILABILITY STATEMENT

Sequences for *C. fulva* have been deposited in GenBank as Accession Numbers MT894435-MT894470 (ITS), MT914511-MT914554 (trnT-trnL), MT914555-MT914601 (trnL intron), MT950637-MT950682 (trnL-trnF), and MT966280-MT966314 (matK2).

AUTHOR CONTRIBUTIONS

RR initiated the study. GB and AL conducted the field and molecular research. AD and JG analyzed UV photography and flower color signals. All authors contributed to and agreed with the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.599874/full#supplementary-material

Supplementary Figure 1 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by nuclear ITS sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−1231.23) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 608 positions in the final dataset.

Supplementary Figure 2 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by chloroplast trnT-trnL sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−1865.73) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Supplementary Figure 3 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by chloroplast trnL-trnL sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−270.23) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 142 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Supplementary Figure 4 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by chloroplast trnL-trnF sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−6092.21) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 519 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Supplementary Figure 5 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by chloroplast matK2 (1571f-trnK2r) sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−1562.42) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 37 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 767 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Supplementary Figure 6 | Phylogenetic relationships of *Caladenia* species with polymorphic flower color collected at Deep Lead as shown by concatenating sequences of nuclear ribosomal DNA (internal transcribed spacer region) with four sequences of chloroplast DNA (three regions in trnT-trnF and matK2 (1571f-trnK2r) region). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The

tree with the highest log likelihood (−12,191.78) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 2691 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

REFERENCES

Alexandersson, R., and Agren, J. (1996). Population size, pollinator visitation and fruit production in the deceptive orchid *Calypso bulbosa*. *Oecologia* 107, 533–540. doi: 10.1007/bf00333945

Australian Government Bureau of Meteorology. (2020). *Climate statistics for Australian locations. Data for weather station 079105*. Available online at: http://www.bom.gov.au/climate/averages/tables/cw_079105.shtml (accessed August 9, 2020).

Australian Government Department of Agriculture Water and the Environment (2018). *Species profile and threats database*. Available online at: http://www.environment.gov.au/cgi-bin/sprat/public/publicthreatenedlist.pl?wanted=flora (accessed August 9, 2020).

Backhouse, G., and Jeanes, J. (1995). *The Orchids of Victoria. Carlton*. Victoria: The Miegunyah Press.

Baguette, M., Bertrand, J. A. M., Stevens, V. M., and Schatz, B. (2020). Why are there so many bee-orchid species? Adaptive radiation by intraspecific competition for mnesic pollinators. *Biol. Rev.* in press doi: 10.1111/brv.12633

Bischoff, M., Lord, J. M., Robertson, A. W., and Dyer, A. G. (2013). Hymenopteran pollinators as agents of selection on flower colour in the New Zealand mountains: salient chromatic signals enhance flower discrimination. *N.Z. J. Bot.* 51, 183–193. doi: 10.1080/0028825X.2013.806933

Bohman, B., Phillips, R. D., Flematti, G. R., Barrow, R. A., and Peakall, R. (2017). The spider orchid *Caladenia crebra* produces sulfurous pheromone mimics to attract its male wasp pollinator. *Angewand. Chem.* 12, 8575–8578. doi: 10.1002/ange.201702864

Bohman, B., Phillips, R. D., Flematti, G. R., and Peakall, R. (2018). (Methylthio)phenol semiochemicals are exploited by deceptive orchids as sexual attractants for *Campylothynnus* thynnine wasps. *Fitoterapia* 126, 78–82. doi: 10.1016/j.fitote.2017.09.022

Bower, C. C. (1996). Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of *Chiloglottis* (Orchidaceae : Caladeniinae). *Aust. J. Bot.* 44, 15–33. doi: 10.1071/bt9960015

Bower, C. C., and Brown, G. R. (2009). Pollinator specificity, cryptic species and geographical patterns in pollinator responses to sexually deceptive orchids in the genus *Chiloglottis*: the *Chiloglottis gunnii* complex. *Aust. J. Bot.* 57, 37–55. doi: 10.1071/bt08164

Breitkopf, H., Onstein, R. E., Cafasso, D., Schlüter, P. M., and Cozzolino, S. (2015). Multiple shifts to different pollinators fuelled rapid diversification in sexually deceptive *Ophrys* orchids. *N. Phytol.* 207, 377–389. doi: 10.1111/nph.13219

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510.

Carr, G. W. (1991). New taxa in *Caladenia* R.Br., *Chiloglottis* R.Br. and *Gastrodia* R.Br. (Orchidaceae) from south eastern Australia. *Indig. Flora Fauna Assoc. Mis. Pap.* 1:7.

Carr, G. W., McMahon, A. R. G., and Todd, J. A. (1989). Vegetation communities and vascular flora of Deep Lead Flora and Fauna Reserve, near Stawell, Victoria: an assessment of their significance and the potential impact of exploratory drilling in the area. Clifton Hill, Victoria: Ecol. Hort. Pty Ltd.

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543.

Clements, M. A., Howard, C. G., and Miller, J. T. (2015). *Caladenia* revisited: results of molecular phylogenetic analyses of Caladeniinae plastid and nuclear loci. *Am. J. Bot.* 102, 581–597. doi: 10.3732/ajb.1500021

Coates, F., and Duncan, M. (2009). Demographic variation between populations of *Caladenia orientalis* – a fire-managed threatened orchid. *Aust. J. Bot.* 57, 326–339. doi: 10.1071/bt08144

Coates, F., Jeanes, J., and Pritchard, A. (2002). *Recovery plan for twenty-five threatened orchid taxa of Victoria, South Australia and New South Wales 2003-2007 (as Arachnorchis fulva). Department of Sustainability and Environment*. Available online from: http://www.environment.gov.au/biodiversity/threatened/recovery-plans/national-recovery-plan-twenty-five-threatened-orchid-taxa-victoria-south-australia-and-new (Verified 20 July 2020).

Coates, F., Lunt, I. D., and Tremblay, R. L. (2006). Effects of disturbance on population dynamics of the threatened orchid *Prasophyllum correctum* D.L. Jones and implications for grassland management in south-eastern Australia. *Biol. Cons.* 129, 59–69. doi: 10.1016/j.biocon.2005.06.037

Delle-Vedove, R., Schatz, B., and Dufay, M. (2017). Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Ann. Bot.* 120, 1–20. doi: 10.1093/aob/mcx055

Dormont, L., Delle-Vedove, R., Bessière, J.-M., and Schatz, B. (2014). Floral scent emitted by white and coloured morphs in orchids. *Phytochemistry* 100, 51–59. doi: 10.1016/j.phytochem.2014.01.009

Dormont, L., Joffard, N., and Schatz, B. (2019). Intraspecific variation in floral color and odor in orchids. *Int. J. Plant Sci.* 180, 1036–1058. doi: 10.1086/705589

Dyer, A. G. (1998). The colour of flowers in spectrally variable illumination and insect pollinator vision. *J. Comp. Physiol. A* 183, 203–212. doi: 10.1007/s003590050248

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. Lond. B* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Garcia, J. E., van der Kooi, C. J., and Wong, B. B. M. (2019). Colour preferences of *Tetragonula carbonaria* Sm. stingless bees for colour morphs of the Australian native orchid *Caladenia carnea*. *J. Comp. Physiol. A* 205, 347–361. doi: 10.1007/s00359-019-01346-0

Dyer, A. G., and Murphy, A. H. (2009). Honeybees choose" incorrect" colors that are similar to target flowers in preference to novel colors. *Isr. J. Plant Sci.* 57, 203–210. doi: 10.1560/ijps.57.3.203

Elmeer, K., Sarwath, H., Malek, J., Baum, M., and Hamwieh, A. (2011). New microsatellite markers for assessment of genetic diversity in date palm (*Phoenix dactylifera* L.). *3 Biotech* 1, 91–97. doi: 10.1007/s13205-011-0010-z

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evol.* 39, 783–791. doi: 10.2307/2408678

Floyd, R., Abebe, E., Papert, A., and Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Mol. Ecol.* 113:839e850.

Garcia, J. E., Shrestha, M., and Dyer, A. G. (2018). Flower signal variability overwhelms receptor-noise and requires plastic color learning in bees. *Behav. Ecol.* 29, 1286–1297.

Garcia, J. E., Spaethe, J., and Dyer, A. G. (2017). The path of colour discrimination is S-shaped: behaviour determines the interpretation of colour models. *J. Comp. Physiol. A* 203, 983–997. doi: 10.1007/s00359-017-1208-2

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers – colour as a perception not a trait. *Front. Plant Sci.* 2020:601700. doi: 10.3389/fpls.2020.601700

Gaskett, A. C. (2011). Orchid pollination by sexual deception: pollinator perspectives. *Biol. Rev.* 86, 33–75. doi: 10.1111/j.1469-185x.2010.00134.x

Gaskett, A. C., Endler, J. A., and Phillips, R. D. (2017). Convergent evolution of sexual deception via chromatic and achromatic contrast rather than colour mimicry. *Evol. Ecol.* 31, 205–227. doi: 10.1007/s10682-016-9863-2

Gaskett, A. C., and Herberstein, M. E. (2010). Colour mimicry and sexual deception by Tongue orchids (Cryptostylis). *Naturwiss* 97, 97–102. doi: 10.1007/s00114-009-0611-0

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2001). Negative freqency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. *Proc. Natl. Acad. Sci. USA* 98, 6253–6255. doi: 10.1073/pnas.111162598

Gill, D. E. (1989). "Fruiting failure, pollinator inefficiency, and speciation in orchids," in *Speciation and its Consequences*, eds D. One and J. A. Endler (Sunderland MA):SinauerAssociates.

Gregg, K. B. (1989). Reproductive biology of the orchid *Cleistes varicata* (L.) Ames var. *bifaria* Fernald growing in a West Virginia meadow. *Castanea* 54, 57–78.

Hartl, D. L., and Clarke, A. G. (2007). *Principles of Population Genetics*. Sunderland, MA: Sinauer.

Hopper, S. D. (2009). Taxonomic turmoil down under: recent developments in Australian orchid systematics. *Ann. Bot.* 104, 447–455. doi: 10.1093/aob/mcp090

Hopper, S. D., and Brown, A. P. (2004). Robert Brown's *Caladenia* revisited, including a revision of its sister genera *Cyanicula, Ericksonella* and *Pheladenia* (Caladeniinae: Orchidaceae). *Aust. Syst. Bot.* 17, 171–240. doi: 10.1071/sb03002

Howard, S. R., Avarguès-Weber, A. A., Garcia, J. E., Greentree, A. D., and Dyer, A. G. (2019). Numerical cognition in honeybees enables addition and subtraction. *Sci. Adv.* 5:eaav0961. doi: 10.1126/sciadv.aav0961

Huelsenbeck, J. P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755. doi: 10.1093/bioinformatics/17.8.754

Huxley, J. S. (1955). Morphism and evolution. *Heredity* 9, 1–51. doi: 10.1038/hdy.1955.1

Huynh, T. (1999). *In-vitro* propagation of the endangered Gaping Leek Orchid, *Prasophyllum correctum*. Honours thesis, Melbourne: RMIT University.

Jeanes, J. (2015). ""*Caladenia fulva* G.W. Carr"," in *Flora of Victoria Vol. 2. Ferns and Allied Plants, Conifers and Monocotyledons*, eds N. G. Walsh and T. J. Entwisle (Melbourne: Inkata Press). Available online at: https://vicflora.rbg.vic.gov.au/flora/taxon/f1ecaf7c-d199-4f5c-98b5-a79cc0c047a5 (accessed August 9, 2020).

Jersáková, J., Johnson, S. D., and Kindlmann, P. (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biol. Rev.* 81, 219–235. doi: 10.1017/s1464793105006986

Jiménez-López, F., Matas, L., Arista, M., and Ortiz, P. L. (2020). Flower colour segregation and flower discrimination under the bee vision model in the polymorphic *Lysimachia arvensis*. *Plant Biosystems* 154, 535–543. doi: 10.1080/11263504.2019.1651776

Jin, W.-T., Shuiteman, A., Chase, M. W., Li, J.-W., Chung, S.-W., Hsu, T.-C., et al. (2017). Phylogenetics of subtribe Orchidinae s.l. (Orchidaceae; Orchidoideae) based on seven markers (plastid matK, psaB, rbcL, trnL-F, trnH-psba, and nuclear nrITS, Xdh): implications for generic delimitation. *BMC Plant Biol.* 17:222. doi: 10.1186/s12870-017-1160-x

Joffard, N., Massol, F., Grenié, M., Montgelard, C., and Schatz, B. (2019). Effect of pollination strategy, phylogeny and distribution on pollination niches of Euro-Mediterranean orchids. *J. Ecol.* 107, 478–490. doi: 10.1111/1365-2745.13013

Jones, D. L. (2008). *A Complete Guide to Native Orchids of Australia, including the Island Territories*. Sydney: Reed New Holland.

Jones, D. L., Clements, M. A., Sharma, I. K., and Mackenzie, A. M. (2001). A new classification of *Caladenia* R.Br. (Orchidaceae). *Orchadian Sept.* 2001:389.

Judd, D. B., MacAdam, D. L., Wyszecki, G., Budde, H. W., Condit, H. R., Henderson, S. T., et al. (1964). Spectral distribution of typical daylight as a function of correlated color temperature. *J. Opt. Soc. Am.* 54:1031. doi: 10.1364/josa.54.001031

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1:252. doi: 10.1016/1360-1385(96)20008-1

Kevan, P. G., Chittka, L., and Dyer, A. (2001). Limits to the salience of ultraviolet: lessons from colour vision in birds and bees. *J. Exp. Biol.* 204, 2571–2580.

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Stahl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120. doi: 10.1663/0006-8101(2006)72[1:DADOFS]2.0.CO;2

Kuiter, R. (2016). *Orchid Pollinators of Victoria, 4th edn*. Seaford: Aquatic Photographics.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Majetic, C. J., Raguso, R. A., Tonsor, S. J., and Ashman, T. L. (2007). Flower color-flower scent associations in polymorphic *Hesperis matronalis* (Brassicaceae). *Phytochemistry* 68, 865–874. doi: 10.1016/j.phytochem.2006.12.009

Martens, S., Knott, J., Seitz, C. A., Janvari, L., Yu, S.-N., and Forkmann, G. (2003). Impact of biochemical pre-studies on specific metabolic engineering strategies of flavonoid biosynthesis in plant tissues. *Biochem. Eng. J.* 14, 227–235. doi: 10.1016/s1369-703x(02)00224-3

Mol, J., Grotewold, E., and Kees, R. (1998). How genes paint flowers and seeds. *Trends Plant Sci.* 3, 212–217. doi: 10.1016/s1360-1385(98)01242-4

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20(Suppl. 1), 8–20. doi: 10.1111/plb.12575

Nilsson, L. A. (1979). Anthecological studies on the Lady's Slipper, *Cypripedium calceolus* (Orchidaceae). *Bot. Not.* 132, 329–347.

Paulus, H. F. (2018). Pollinators as isolation mechanisms: field observations and field experiments regarding specificity of pollinator attraction in the genus *Ophrys* (Orchidaceae). *Entomol. Gen.* 37, 261–316. doi: 10.1127/entomologia/2018/0650

Paulus, H. F. (2019). Speciation, pattern recognition and the maximization of pollination: general questions and answers given by the reproductive biology of the orchid genus *Ophrys*. *J. Comp. Physiol. A* 205, 285–300. doi: 10.1007/s00359-019-01350-4

Peakall, R. (1990). Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. *Funct. Ecol.* 4, 159–167. doi: 10.2307/2389335

Peakall, R., and Beattie, A. J. (1996). Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. *Evol* 50, 2207–2220. doi: 10.2307/2410692

Peakall, R., and Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mole. Ecol. Notes* 6, 288–295. doi: 10.1111/j.1471-8286.2005.01155.x

Peakall, R., and Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* 28, 2537–2539. doi: 10.1093/bioinformatics/bts460

Peakall, R., and Whitehead, M. R. (2014). Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids. *Ann. Bot.* 113, 341–355. doi: 10.1093/aob/mct199

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectal input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Peter, C. I., and Johnson, S. D. (2008). Mimics and magnets: the importance of color and ecological facilitation in floral deception. *Ecol.* 89, 1583–1595. doi: 10.1890/07-1098.1

Phillips, R. D., Backhouse, G., Brown, A. P., and Hopper, S. D. (2009b). Biogeography of *Caladenia* (Orchidaceae), with special reference to the South-west Australian Floristic Region. *Aust. J. Bot.* 57, 259–275. doi: 10.1071/bt08157

Phillips, R. D., Bohman, B., Brown, G. R., Tomlinson, S., and Peakall, R. (2020). A specialised pollination system using nectar-seeking thynnine wasps in *Caladenia nobilis*. *Plant Biol.* 22, 157–166. doi: 10.1111/plb.13069

Phillips, R. D., Brown, G. R., Dixon, K. W., Hayes, C., Linde, C. C., and Peakall, R. (2017). Evolutionary relationships among pollinators and repeated pollinator sharing in sexually deceptive orchids. *J. Evol. Biol.* 30, 1674–1691. doi: 10.1111/jeb.13125

Phillips, R. D., Faast, R., Bower, C., Brown, G., and Peakall, R. (2009a). Implications of pollination by food and sexual deception for pollinator specificity, fruit set, population genetics and conservation of *Caladenia* (Orchidaceae). *Aust. J. Bot.* 57, 276–286.

Phillips, R. D., and Peakall, R. (2018). An experimental evaluation of traits that influence the sexual behavious of pollinators in sexually deceptive orchids. *J. Evol. Biol.* 31, 1732–1742. doi: 10.1111/jeb.13370

Primack, R., and Stacy, E. (1998). Cost of reproduction in the Pink Lady's Slipper orchid (*Cypripedium acaule*, Orchidaceae): an eleven-year experimental study of three populations. *Am. J. Bot.* 85, 1672–1679. doi: 10.2307/2446500

Primack, R. B., and Hall, P. (1990). Costs of reproduction in the pink lady's slipper orchid: a four-year experimental study. *Am. Nat.* 136, 638–656. doi: 10.1086/285120

Pritchard, H. W. (1985). Determination of orchid seed viability using fluorescein diacetate. *Plant Cell Environ.* 8, 727–730. doi: 10.1111/j.1365-3040.1985.tb01714.x

Rakosy, D., Streinzer, M., Paulus, H. F., and Spaethe, J. (2012). Floral visual signal increases reproductive success in a sexually deceptive orchid. *Arthropod-Plant Interact.* 6, 671–681. doi: 10.1007/s11829-012-9217-0

Reiter, N., Lawrie, A. C., and Linde, C. C. (2018a). Matching symbiotic associations of an endangered orchid to habitat to improve conservation outcomes. *Ann. Bot.* 122, 947–959.

Reiter, N., Bohman, B., Flematti, G. R., and Phillips, R. D. (2018b). Pollination by nectar-foraging thynnine wasps: evidence of a new specialized pollination system for Australian orchids. *Bot. J. Linn. Soc.* 188, 327–337.

Reiter, N., Bohman, B., Batley, M., and Phillips, R. D. (2019). Pollination of an endangered *Caladenia* species (Orchidaceae) by nectar-foraging behaviour of a widespread species of colletid bee. *Bot. J. Linn. Soc.* 189, 83–98. doi: 10.1093/botlinnean/boy074

Ryberg, A., Olsson, C., Ahrné, S., and Monstein, H.-J. (2011). Comparison of $(GTG)_5$-oligonucleotide and ribosomal intergenic transcribed spacer (ITS)-PCR for molecular typing of *Klebsiella* isolates. *J. Microbiol. Meth.* 84, 183–188. doi: 10.1016/j.mimet.2010.11.019

Sauquet, H., Doyle, J. A., Scharaschkin, T., Borsch, T., Hilu, K. W., Chatrou, L. W., et al. (2003). Phylogenetic analysis of Magnoliales and Myristicaceae based on multiple data sets: implications for character evolution. *Bot. J. Linn. Soc.* 142, 125–186. doi: 10.1046/j.1095-8339.2003.00171.x

Scaccabarozzi, D., Cozzolino, S., Guzzetti, L., Galimberti, A., Milne, L., Dixon, K. W., et al. (2018). Masquerading as pea plants: behavioural and morphological evidence for mimicry of multiple models in an Australian orchid. *Ann. Bot.* 122, 1061–1073.

Schatz, B., Gauthier, P., Debussche, M., and Thompson, J. (2014). A decision tool for listing species for protection on different geographic scales and administrative levels. *J. Nat. Cons.* 22, 75–83. doi: 10.1016/j.jnc.2013.09.003

Schiestl, F. P. (2005). On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* 92, 255–264. doi: 10.1007/s00114-005-0636-y

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Schiestl, F. P., and Schluter, P. M. (2009). Floral isolation, specialized pollination, and pollinator behavior in orchids. *Ann. Rev. Entomol.* 54, 425–446. doi: 10.1146/annurev.ento.54.110807.090603

Sharma, I. K., Clements, M. A., and Jones, D. L. (2000). Observations of high genetic variability in the endangered Australian terrestrial orchid *Pterostylis gibbosa* R.Br. (Orchidaceae). *Biochem. Syst. Ecol.* 28, 651–663. doi: 10.1016/s0305-1978(99)00107-6

Shrestha, M., Dyer, A. G., Dorin, A., Ren, Z.-X., and Burd, M. (2020). Rewardlessness in orchids: how frequent and how rewardless? *Plant Biol.* 2020:13113. doi: 10.1111/plb.13113

Scopece, G., Cozzolino, S., Johnson, S. D., and Schiestl, F. P. (2010). Pollination efficiency and the evolution of specialized deceptive pollination systems. *Am. Nat.* 175, 98–105. doi: 10.1086/648555

Scopece, G., Schiestl, F. P., and Cozzolino, S. (2014). Pollen transfer efficiency and its effect on inflorescence size in deceptive pollination strategies. *Plant Biol.* 17, 545–550. doi: 10.1111/plb.12224

Smithson, A., and Macnair, M. R. (1997). Negative frequency-dependent selection by pollinators on artificial flowers without rewards. *Evolution* 51, 715–723. doi: 10.1111/j.1558-5646.1997.tb03655.x

Snow, A. A., and Whigham, D. E. (1989). Costs of flower and fruit production in *Tipilaria discolor* (Orchidaceae). *Ecol.* 70, 1286–1293. doi: 10.2307/1938188

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Spaethe, J., Streinzer, M., and Paulus, H. F. (2010). Why sexually deceptive orchids have colored flowers? *Comm. Integ. Biol.* 3, 139–141. doi: 10.4161/cib.3.2.10333

Steane, D. A., Wilson, K. L., and Hill, R. S. (2003). Using matK sequence data to unravel the phylogeny of Casuarinaceae. *Mol. Phylogen. Evol.* 28, 47–59. doi: 10.1016/s1055-7903(03)00028-9

Stejskal, K., Streinzer, M., Dyer, A., Paulus, H. F., and Spaethe, J. (2015). Functional significance of labellum pattern variation in a sexually deceptive orchid (*Ophrys heldreichii*): evidence of individual signature learning effects. *PLoS One* 10:e0142971. doi: 10.1371/journal.pone.0142971

Stoutamire, W. P. (1983). Wasp pollination species of *Caladenia* (Orchidaceae) in south-western Australia. *Aust. J. Bot.* 31, 383–394. doi: 10.1071/bt9830383

Streinzer, M., Ellis, T., Paulus, H. F., and Spaethe, J. (2010). Visual discrimination between two sexually deceptive *Ophrys* species by a bee pollinator. *Arthropod-Plant Interactions* 4, 141–148. doi: 10.1007/s11829-010-9093-4

Streinzer, M., Paulus, H. F., and Spaethe, J. (2009). Floral colour signal increases short-range detectability of a sexually deceptive orchid. *J. Exp. Biol.* 212, 1365–1370. doi: 10.1242/jeb.027482

Swarts, N. D., Clements, M. A., Bower, C. C., and Miller, J. T. (2014). Defining conservation units in a complex of morphologically similar, sexually deceptive, highly endangered orchids. *Biol. Cons.* 174, 55–64. doi: 10.1016/j.biocon.2014.03.017

Taberlet, P., Gielly, L., Pautou, G., and Bouvet, J. (1991). Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Mol. Biol.* 17, 1105–1109. doi: 10.1007/bf00037152

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10, 512–526.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). Clustal W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc. Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Threatened Species Scientific Committee (2016). *Conservation advice: Caladenia fulva* (Tawny Spider-orchid). Canberra: Department of the Environment, Australian Government. Available online at: http://www.environment.gov.au/biodiversity/threatened/species/pubs/24371-conservation-advice-16122016.pdf (accessed November 15, 2020).

Tremblay, R. L., Ackerman, J. D., Zimmerman, J. K., and Calvo, R. N. (2005). Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biol. J. Linn. Soc.* 84, 1–54. doi: 10.1111/j.1095-8312.2004.00400.x

Tull, T. (1998). Fruit set and recruitment in populations of *Cypripedium calceolus* L. in Estonia. *J. Linn. Soc.* 126, 27–38. doi: 10.1111/j.1095-8339.1998.tb02513.x

Vandewoestijne, S., Róis, A. S., Caperta, A., Baguette, M., and Tyteca, D. (2009). Effects of individual and population parameters on reproductive success in three sexually deceptive orchid species. *Plant Biol.* 11, 454–463. doi: 10.1111/j.1438-8677.2008.00125.x

Vereecken, N. J., Cozzolino, S., and Schiestl, F. P. (2010). Hybrid floral scent novelty drives pollinator shift in sexually deceptive orchids. *BMC Evol. Biol.* 10:103. doi: 10.1186/1471-2148-10-103

Victorian Government Department of Environment, Land, Water and Planning (2019). *Protected Flora List November 2019*. Available online at: https://www.environment.vic.gov.au/__data/assets/pdf_file/0011/50420/20191114-FFG-protected-flora-list.pdf (accessed August 9, 2020).

Victorian Government Department of Environment, Land, Water and Planning (2020). *Nature Kit*. URL: http://maps.biodiversity.vic.gov.au/viewer/?viewer=NatureKit (accessed August 9, 2020).

von Helversen, O. (1972a). "The relationship between difference in stimuli and choice frequency in training experiments with the honeybee," in *Information processing in the visual systems of anthropods*, ed. R. Wehner (Berlin Heidelberg: Springer), 323–334. doi: 10.1007/978-3-642-65477-0_46

von Helversen, O. (1972b). Zur spektralen Unterschiedsempfindlichkeit der Honigbiene. *J. Comp. Physiol. A* 80, 439–472. doi: 10.1007/bf00696438

Waite, S., and Farrell, L. (1998). Population biology of the rare military orchid (*Orchis militaris* L.) at an established site in Suffolk. *England. Bot. J. Linn. Soc.* 126, 109–126. doi: 10.1111/j.1095-8339.1998.tb02520.x

Waite, S., Hopkins, N., and Hitchings, S. (1991). "Levels of pollinia export, import and fruit production among plants of *Anacamptis pyramidalis*, *Dactylorhiza fichsii*, and *Epipactis helleborine*," in *Population Ecology of Terrestrial Orchids*, eds T. C. E. Wells and J. H. Willems (The Hague: SBP Academic Publishing), 103–110.

Walsh, N. G., and Entwisle, T. J. (eds) (1994). *Flora of Victoria, Vol. 2. Ferns and Allied Plants, Conifers and Monocotyledons*. Available online at: https://vicflora.rbg.vic.gov.au/ (accessed August 9, 2020).

Weston, P. H., Perkins, A. J., Indsto, J. O., and Clements, M. A. (2014). "Phylogeny of the Orchidaceae tribe Diurideae and its implication for the evolution of

pollination systems," in *Darwin's orchids: then and now*, eds R. Edens-Meier and P. Bernhardt (Chicago: The University of Chicago Press), 91–154.

White, T. J., Bruns, T. D., Lee, S., and Taylor, J. J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR protocols: a guide to methods and applications*, eds M. A. Gelfand, J. J. Sninsky, and J. White (New York,NY: Academic Press), 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1

Willems, J. H., and Melser, C. (1998). Population dynamics and life-history of *Coeloglossum viride* (L.) Hartm.: an endangered orchid species of the Netherlands. *Bot. J. Linn. Soc.* 126, 83–93. doi: 10.1111/j.1095-8339.1998.tb02518.x

Wong, B. B. M., Salzmann, C., and Schiestl, F. P. (2004). Pollinator attractiveness increases with distance from flowering orchids. *Proc. R. Soc. Lond. B Biol. Sci.* 271, S212–S214.

Wong, B. B. M., and Schiestl, F. P. (2002). How an orchid harms its pollinator. *Proc. R. Soc. Lond. B Biol. Sci.* 269, 1529–1532. doi: 10.1098/rspb.2002.2052

Wong, C. J., Pichersky, E., and Peakall, R. (2017). The biosynthesis of unusual floral volatiles and blends involved in orchid pollination by deception: current progress and future prospects. *Front. Plant Sci.* 8:1955. doi: 10.3389/fpls.2017.01955

Wraith, J., and Pickering, C. (2019). A continental scale analysis of threats to orchids. *Biol. Cons.* 234, 7–17. doi: 10.1016/j.biocon.2019.03.015

Wright, M., Cross, R., Dixon, K., Huynh, T., Lawrie, A. C., Nesbit, L., et al. (2009). Propagation and reintroduction of *Caladenia*. *Aus. J. Bot.* 57, 373–387. doi: 10.1071/bt08137

Wyszecki, G., and Stiles, W. S. (1982). *Color Science Concepts and Methods, Quantitative Data and Formulae*, 2nd Edn. New York,NY: John Wiley & Sons.

Zimmerman, J. K., and Aide, T. M. (1989). Patterns of fruit production in a neotropical orchid: pollination vs. resource limitation. *Am. J. Bot.* 76, 67–73. doi: 10.1002/j.1537-2197.1989.tb11286.x

Zuker, A., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., et al. (2002). Modification of flower colour and fragrance by antisense suppression of the flavonone 3-hydroxylase gene. *Mol. Breed.* 9, 33–41. doi: 10.1023/A:1019204531262

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Flowering Phenology and the Influence of Seasonality in Flower Conspicuousness for Bees

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Flowering patterns are crucial to understand the dynamics of plant reproduction and resource availability for pollinators. Seasonal climate constrains flower and leaf phenology, where leaf and flower colors likely differ between seasons. Color is the main floral trait attracting pollinators; however, seasonal changes in the leaf-background coloration affect the perception of flower color contrasts by pollinators. For a seasonally dry woody cerrado community (Brazilian savanna) mainly pollinated by bees, we verified whether seasonality affects flower color diversity over time and if flower color contrasts of bee-pollinated species differ between seasons due to changes in the leaf-background coloration. For 140 species, we classified flower colors based on human-color vision, and for 99 species, we classified flower colors based on bee-color vision (spectral measurements). We described the community's flowering pattern according to the flower colors using a unique 11 years phenological database. For the 43 bee-pollinated species in which reflectance data were also available, we compared flower color diversity and contrasts against the background between seasons, considering the background coloration of each season. Flowering was markedly seasonal, peaking at the end of the dry season (September), when the highest diversity of flower colors was observed. Yellow flowers were observed all year round, whereas white flowers were seasonal, peaking during the dry season, and pink flowers predominated in the wet season, peaking in March. Bee-bluegreen flowers peaked between September and October. Flowers from the wet and dry seasons were similarly conspicuous against their corresponding background. Regardless of flowering season, the yellowish background of the dry season promoted higher flower color contrast for all flower species, whereas the greener background of the wet season promoted a higher green contrast. Temporal patterns of flower colors and color contrasts were related to the cerrado seasonality, but also to bee's activity, visual system, and behavior. Background coloration affected flower contrasts, favoring flower conspicuousness to bees according to the season. Thus, our results provide new insights regarding the temporal patterns of plant–pollinator interactions.

Keywords: background coloration, bee visual system, cerrado *sensu stricto*, color contrasts, flower color diversity, flowering patterns, plant–animal communication, plant community

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INTRODUCTION

In tropical ecosystems, seasonal changes in rainfall, temperature, and day length are the primary flowering constraints (Morellato et al., 2000; Abrahamczyk et al., 2011; Cortés-Flores et al., 2017). Seasonality shapes flowering patterns, which are strongly related to the dynamics of plant communities, defining temporal changes in plant reproduction, resource availability for flower visitors, maintenance of plant–pollinator diversity, and plant reproductive success (Gentry, 1974; Lieth, 1974; Ramírez, 2006; Morellato et al., 2016). Thus, concentrating flowering in periods with favorable weather conditions could increase pollinators' diversity and activity (Frankie et al., 1974; Newstrom et al., 1994; Ramírez, 2006). Additionally, flowering patterns are also shaped by biotic factors, mainly plant–pollinator interactions (Ramírez, 2006; Cortés-Flores et al., 2017). Pollinators exert a key selective pressure on flowering, affecting the intensity, productivity, and length of the reproductive season and also species' synchronicity (Augspurger, 1981, 1983; Smith-Ramírez et al., 1998).

Flowers attract pollinators by distinct stimuli, such as color, shape, size, and scent (Fenster et al., 2004). However, color is the main floral trait related to pollinator attraction and plant–animal communication; flower color is adapted to pollinators' visual sensitivity and preferences (Lunau and Maier, 1995; Chittka et al., 1999; Fenster et al., 2004; Dyer et al., 2006). Hence, the diversity of flower colors in angiosperms is mainly a consequence of the selective pressures exerted by pollinators (Chittka and Menzel, 1992; Dyer et al., 2012; Shrestha et al., 2013; Camargo et al., 2019). Bees are the dominant pollinators in several plant communities and biomes around the world, mainly due to their large morphological variation, distinct foraging behaviors, total dependency on floral resources, and highly developed communication system (Ollerton, 2017). Although Hymenoptera are active throughout the year, a higher activity, especially in bees, has been observed mainly between November and June in cerrado woodland (D'Avila and Marchini, 2008). Eusocial bees, such as *Apis mellifera* and *Trigona spinipes*, are less susceptible to temperature and humidity variation, being important pollinators during drier periods when less resources are available (D'Avila and Marchini, 2008).

Bees, as most Hymenoptera, possess a trichromatic color vision and perceive colors based on photoreceptors sensible to green, blue, and ultraviolet (UV) wavelengths (Chittka and Menzel, 1992). In general, bees prefer flowers that reflect short wavelengths and saturated colors (higher spectral purity), where the contrast against the background is a key signal for flower distinction and detection (Rohde et al., 2013; Telles et al., 2017; Camargo et al., 2019).

The background promoting flower contrast is composed mainly by leaves that change colors throughout the year in seasonal ecosystems (Alberton et al., 2014; Camargo et al., 2014). In tropical seasonally dry forests, most species are deciduous or semi-deciduous, losing their leaves in the dry season (Alberton et al., 2014, 2019; Camargo et al., 2014, 2018). During senescence, leaves lose chlorophyll, altering the background color from green (formed by young and mature leaves in the rainy season) to yellowish, brownish, or reddish in the dry season (Camargo et al., 2014). Thus, differences in the background color may affect the flower color contrast and, consequently, flower detection and discrimination by bees (Camargo et al., 2014; Binkenstein and Schaefer, 2015; Bukovac et al., 2017a; Telles et al., 2017).

Using a community-level approach and the cerrado as a model of a tropical seasonally dry vegetation, we investigated the seasonality of flowering phenology, the flower color diversity, and the respective flower color contrasts according to the visual system of bees. Specifically, we asked: (i) Does seasonality influence the diversity of flower colors over time? (ii) Do the color contrasts of bee-pollinated flowers differ between seasons according to changes in the background coloration? (iii) Are distinct bee-flower colors and contrasts over seasons enhancing flower conspicuousness? Flowers are observed throughout the year in the cerrado; however, flowering phenology is seasonal, peaking at the end of the dry season (Batalha and Martins, 2004; Gottsberger and Silberbauer-Gottsberger, 2006; pers. obs). Therefore, we expected the highest diversity of flower colors during the dry season, a period that favors flowering and bees' activity (Morellato and Leitao-Filho, 1996; Gottsberger and Silberbauer-Gottsberger, 2006). We also expected that bee-flower colors and its associated visual contrasts shift between seasons to increase flower contrast against the background of the corresponding flowering season.

MATERIALS AND METHODS

Study Area

The Brazilian cerrado is a Neotropical savanna that contains different vegetation types, from grasslands to woodlands, with high ecological and landscape diversity under a seasonally dry climate (Klink and Machado, 2005; Coutinho, 2006).

Our study was carried out in a cerrado woodland located in Itirapina, São Paulo State, southeastern Brazil (22°10′31.41?S, 47°52′26.13″W). The vegetation of the study area is mainly the cerrado *sensu stricto* (Reys et al., 2013), hereafter called cerrado, which is dominated by woody species and is the typical and most widespread savanna physiognomy of the Brazilian cerrado (Coutinho, 2006; **Supplementary Figures S1A,B**). The vegetation is classified as semi-deciduous and is characterized by a continuous herbaceous layer, scattered shrubs, and a discontinuous tree cover that reaches from 6 to 12 m high (Reys et al., 2013; Camargo et al., 2018; **Supplementary Figure S1C)**. In the cerrado, flowering is seasonal, and most woody species are pollinated by bees (Oliveira and Gibbs, 2002; Gottsberger and Silberbauer-Gottsberger, 2006). The seasonal climate is marked by a rainy warm season from October to March and a dry and cooler season from April to September (Camargo et al., 2018). The mean annual temperature is 20°C, and the annual total rainfall is 1,524 mm (Camargo et al., 2018). The studied cerrado community shows a high diversity of shrubs and trees, with the most species-rich families being Myrtaceae, Fabaceae, Malpighiaceae, and Vochysiaceae and the most abundant species being *Bauhinia rufa* (Bong.) Steudel, *Xylopia aromatica* (Lam.)

Mart., *Miconia rubiginosa* (Bonpl.) A.D., *Virola sebifera* (Aubl), and *Myrcia guianensis* (Aubl.) DC. (Reys et al., 2013).

Plant Survey and Phenology Monitoring

The studied species were selected from a list of 222 woody plant species sampled during a floristic survey carried out every month at the study site since 2004 (Reys et al., 2013). The systematic plant survey was conducted in 36 25 × 2 m transects 50 m apart from each other, where all woody plants with a circumference above the ground >3 cm were tagged, sampled, identified to species level, and monitored individually for reproductive and vegetative phenology (Reys et al., 2013; Camargo et al., 2018; Escobar et al., 2018).

We collected flower reflectance data at least once a month from April 2017 to April 2018, for which we searched and sampled flowering species throughout the study area. We analyzed 140 animal-pollinated plant species previously surveyed (see above) belonging to 97 genera and 44 families (**Supplementary Table S1**). The most representative families were Fabaceae (15%), Bignoniaceae (8.5%), Asteraceae (8%), Malpighiaceae (7%), and Myrtaceae (6%). From the 140 animal-pollinated plant species, 91 were monitored for phenology, and 99 were measured for flower reflectance. However, we only have phenology and spectral measurements for 49 species. We inferred the pollinators for the 140 plant species compiled and found that 115 plant species have bees as the main or secondary pollinator (**Supplementary Table S1**). The pollinators of the studied species were extracted from an extensive review for cerrado pollinators performed by Martins (2019). Vouchers of the plant species sampled are lodged in the Herbarium Rioclarense (HRCB) of the São Paulo State University (UNESP).

Flowering Phenology

Phenological observations have been carried out once a month in the 36 plant systematic survey transects since September 2004 by the Phenology Laboratory team (UNESP—São Paulo State University, campus Rio Claro) as part of the cerrado long-term phenology monitoring program (for details, see Camargo et al., 2013, 2018; Vogado et al., 2016; Escobar et al., 2018). For each observed individual, vegetative (leaf flush and leaf fall) and reproductive (flower buds, anthesis, and unripe and ripe fruits) phenophases are scored using a semi-quantitative index represented by the given classes of intensity: 0 (absence), 1 (≤50% of branches active, intermediate intensity), and 2 (>50% of branches active, peak of intensity) (Opler et al., 1980; Vogado et al., 2016). From the phenology monitoring database, we selected all species that flourished at any given year from January 2005 to December 2015. Hence, we evaluated the temporal patterns of flower color diversity in the cerrado community and related species flowering time to the respective flower color according to human- and bee-color categories, similar to Camargo et al. (2013) in which fruiting patterns were analyzed according to fruit color in the same cerrado area.

Flower Colors and Contrasts

We classified species flower colors according to the human visual system as white (including whitish), yellow, pink (including violet and blue flowers), green, and red (including brown and orange). When available, we used the reflectance spectra to determine the human color using the function *spec2rgb* from the *Pavo* package (Maia et al., 2018) in R (R Development Core Team). For species in which we had the reflectance data, we also classified flower colors according to the visual systems of bees as described below. The low number of species in certain color categories is a consequence of the flower color frequency found for cerrado areas (Oliveira and Gibbs, 2000, 2002; Gottsberger and Silberbauer-Gottsberger, 2006).

We measured the flower spectral reflectance between 300 and 700 nm, including the UV light, using a spectrophotometer (Ocean Optics—Jaz Modular Optical Sensing Suite) equipped with a pulsed xenon light source with a spectral range between 190 and 1,100 nm. The pulsed xenon light avoids the possible degradation of samples by the UV light. To calibrate the equipment and record the reflectance data, we used a PTFE disc (WS-1 Diffuse Reflectance Standard, PTFE—Ocean Optics) as the white standard and a black suede paper as the black standard. Flowers were preserved in individual bags stored in cooler boxes until the measurements were carried out, in the laboratory, on the same day. Flower color was based on the mean reflectance spectra calculated from the reflectance data of 10 flowers (collected from different individuals) for each species (Dalrymple et al., 2015). Flower color was considered as the reflectance spectra of the predominant color in the floral display, which generally corresponded to the color of the petal (Camargo et al., 2019).

To evaluate flower colors and calculate color variables according to the visual systems of bees, we used the bee hexagon proposed by Chittka (1992), which represents the bee-color space. Each photoreceptor present on the retina of the bees' eye (UV, blue, and green) is represented in a vertex of the bee hexagon. Each flower color is represented by a point (color loci) in the hexagon that corresponds to the Euclidian distance between the flower background and the stimulus promoted by the light reflected from the flower in each photoreceptor, according to a previously specified visual system (Chittka, 1992). To calculate the flower color loci in the bee hexagon, we used the visual model of *A. mellifera* proposed by Menzel and Backhaus (1991), the D65 standard daylight, and the reflectance of leaves collected from the plant community in the dry and wet seasons based on Camargo et al. (2013). The leaf background of each season is represented by the mean reflectance spectra composed by leaves of different species collected in November (wet season), when leaves are completely developed after the peak of leaf flushing in September, and in July (dry season) when the peak of leaf fall occurs (Camargo et al., 2013, 2014, 2018). According to the color loci position in the bee hexagon, we classified flower colors in six bee-color categories: bee-blue, bee-green, bee-UV, bee-bluegreen, bee-UVgreen, and bee-UVblue (Chittka et al., 1994).

To analyze differences in the color contrasts between bee-pollinated flowers of the dry and wet seasons, we calculated the color and green contrasts promoted by flowers produced in each season. For such, we calculated the contrasts promoted by flowers produced mainly in the wet season (flowering peak between October and March) against the background of the wet season and calculated the contrasts promoted by flowers

produced during the dry season (flowering peak between April and September) against the background of the dry season. We also calculated and compared the contrasts of flowers produced in the dry and wet seasons against the background of their opposite season. The color contrast or chromatic contrast is the *r* vector in the bee hexagon that is represented by the Euclidian distance between a given flower color loci and the hexagon center, corresponding to the background color locus (Chittka, 1992). The color contrast is important for bees to detect flowers from the background and is activated only at short distances and large visual angles (Spaethe et al., 2001; van der Kooi et al., 2019). The green contrast or achromatic contrast is the contrast between two colors detected by the green photoreceptor and adapted to the background; it is used for long-distance detection and is always active when bees are foraging (Chittka, 1992; Spaethe et al., 2001; van der Kooi et al., 2019). The green contrast against the background corresponds to the green photoreceptor excitation adjusted to the background, that is, the green photoreceptor stimulus subtracted by 0.5 (Spaethe et al., 2001; Dyer et al., 2016).

Data Analyses

We analyzed the flowering phenology using the flower peak date of each species, defined as the most recurrent month with maximum flowering intensity of a given species throughout the 11 years of observations (see Escobar et al., 2018). To test for seasonality in the flowering peak of the community and for each flower color, we used circular statistics based on Morellato et al. (2000, 2010). For such, the flower peak date (month of the year) of each species was converted to an angle or vector direction (15°corresponds to January and so on), after which we calculated the mean angle and angular standard deviation (Morellato et al., 2010) for the community and for each flower color. We then applied the Rayleigh test (Z) to test for the significance of the mean angle or mean date, i.e., whether the species' flower peak dates or angles are significantly concentrated around the mean angle or date (Morellato et al., 2010). If the mean angle is significant, the flower peak pattern is considered seasonal, and the degree of seasonality (of the community or for each flower color) is measured as the length of the mean vector (*r*): the *r* vector ranges from 0, no concentration or no seasonality, to 1, highest concentration around the mean angle or highest degree of seasonality (Morellato et al., 2000, 2010).

We also calculated the linear flowering pattern based on an intensity index calculated by a modified Fournier index (Fournier, 1974) that uses two instead of four classes of intensity (Vogado et al., 2016). This index was calculated for each species as the sum of the intensity classes of the individuals in a given month divided by the total number of individuals of this species multiplied by two, the maximum intensity class (Vogado et al., 2016). For each species, we calculated a unique mean year to represent the flowering pattern using the intensity indices calculated for 11 years. We then calculated the flowering pattern based on this index for the community (all species), for bee-pollinated species, and for species grouped by flower colors according to the human vision.

To compare the color contrasts of bee-pollinated flowers produced mainly during the dry (April to September) and wet (October to March) seasons, we applied a Wilcoxon rank sum test (W) with a continuity correction to compare the quantitative variables of color and green contrasts between the seasons. To verify if seasonal changes in the background coloration interfere with flower conspicuousness, we compared the flower contrasts against the background of the corresponding flowering season and against the background of the opposite season using the Wilcoxon paired signed rank test (V).

Circular statistics were carried out in ORIANA 4.0 (Kovach, 2011) and linear statistics in R (R Development Core Team). In addition, we analyzed the reflectance spectra and calculated the color variables using the *Pavo* package in R (Maia et al., 2018).

RESULTS

Cerrado Plant Pollinators and Flower Colors

Bees were the dominant pollinators: 74% of the 140 plant species were mainly pollinated by bees or 82% if we added plant species that have bees as secondary pollinators (**Supplementary Table S1**).

According to the human eye, white and pink were the most frequent flower colors (52 and 21%, respectively), followed by yellow (17%), green (5%), and red (5%) (**Figure 1A**). Among the 99 species with reflectance data, bee-bluegreen represented half of the species (50%), followed by bee-green (18%), bee-blue (13%), bee-UVgreen (12%), and bee-UVblue (6%) (**Figure 1B**).

Flowering, Color Patterns, Pollination, and Seasonality

Flowering was observed throughout the year in the community based on data of 91 woody animal-pollinated species included in the phenology monitoring. The community flowering peak occurred in September, at the end of the dry season (**Figure 2A** and **Supplementary Figure S2A**), which showed the highest diversity of flowers available for pollinators (29% of species), followed by December (14%) and November (10.5%). From the 91 species analyzed, 83% (75 species) were bee-pollinated (**Supplementary Table S1**). Bee-pollinated flowers were available all year long, but showed a higher availability in September (22 species) and November (13 species) and a lower availability in April, May, and June (only 1 species in each month) (**Figure 2B** and **Supplementary Figure S2B**). The flowering pattern was significantly seasonal (Z = 14.7; p = 0.001; vector r = 0.44) with a mean date in October (**Figure 2B**). For the dataset of 49 species with phenological and reflectance data, the flowering pattern was also significantly seasonal (Z = 13.6; p = 0.001; vector r = 0.52), and the mean date was again in October, peaking in September (**Supplementary Figure S3**).

Flowering phenology showed that white flowers (according to human colors; n = 58) presented a significant seasonal pattern, peaking in September (**Figure 2C** and **Supplementary**

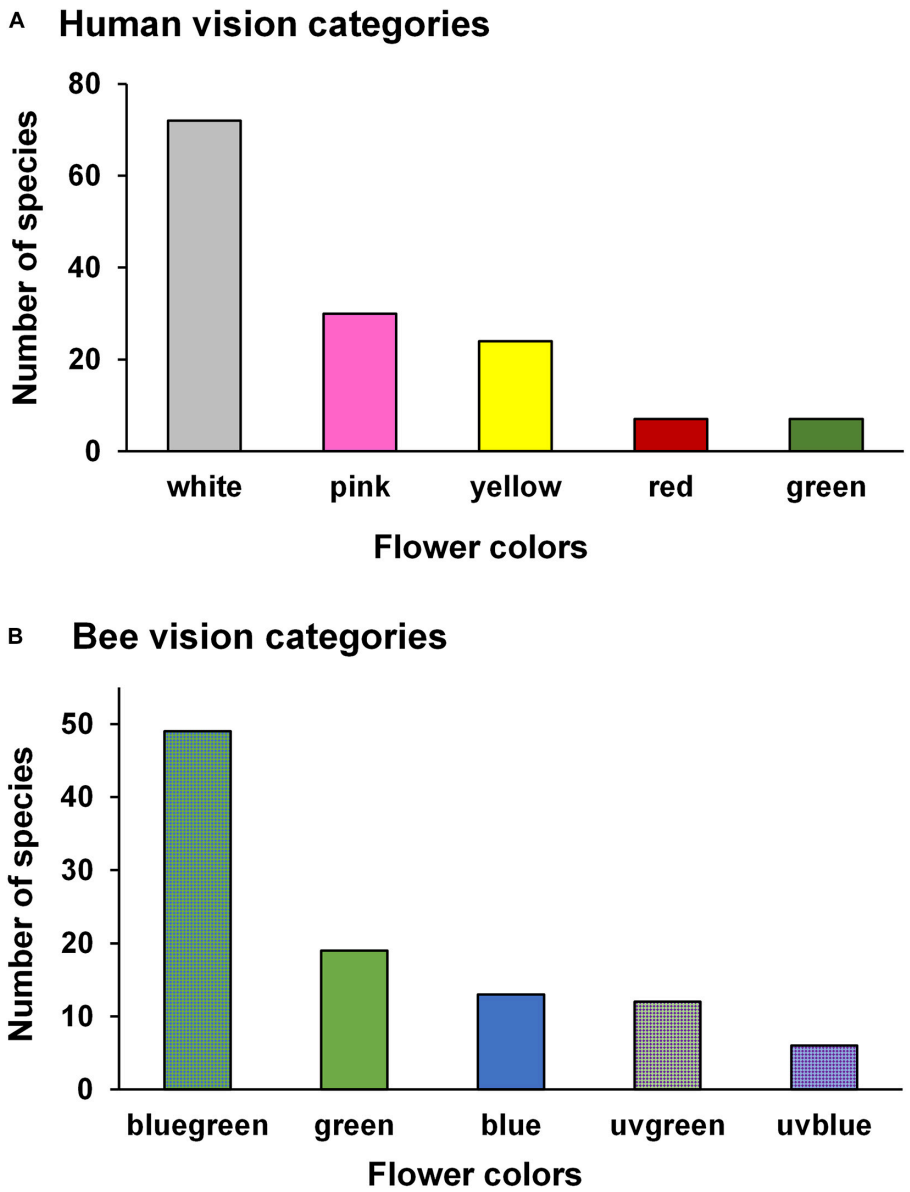


FIGURE 1 | Number of species in each flower color according to the human- **(A)** and bee- **(B)** color vision for 140 and 99 species, respectively, sampled in a woody cerrado. **(A,B)** Include species with different pollinators.

Figure S2C) with a mean date in October (Z = 13.29; $p < 0.001$; vector $r = 0.60$). On the other hand, yellow flowers ($n = 15$) were more distributed along the year, where no peak and no significant seasonality (Z = 0.5; $p = 0.6$) were observed (**Figure 2D** and **Supplementary Figure S2D**). Pink flowers bloomed mainly in the wet season, peaking at the end of the wet season (**Figure 2E** and **Supplementary Figure S2E**) but with no significant seasonality (Z = 0.8; $p = 0.4$). Bees pollinate 86% of white flowers, 90% of pink flowers, and 93% of yellow flowers.

Flowering of bee-bluegreen flowers, the predominant bee color in our community (28 species), was also significantly seasonal, peaking mainly at the end of the dry season (September) with a mean date in October (Z = 8.1; $p = 0.001$; vector $r = 0.53$) (**Figure 2F**). The other bee-color species were not contemplated in our phenological database.

Flower Color and Contrasts in the Dry and Wet Seasons

From the 43 bee-pollinated species for which we had phenological and reflectance data, 22 showed a flowering peak during the rainy season (from October to March; **Figures 3B,E**), whereas 21 peaked during the dry season (from April to September; **Figures 3C,F**). The percentage of bee-bluegreen flowers was similar between seasons: 67% of

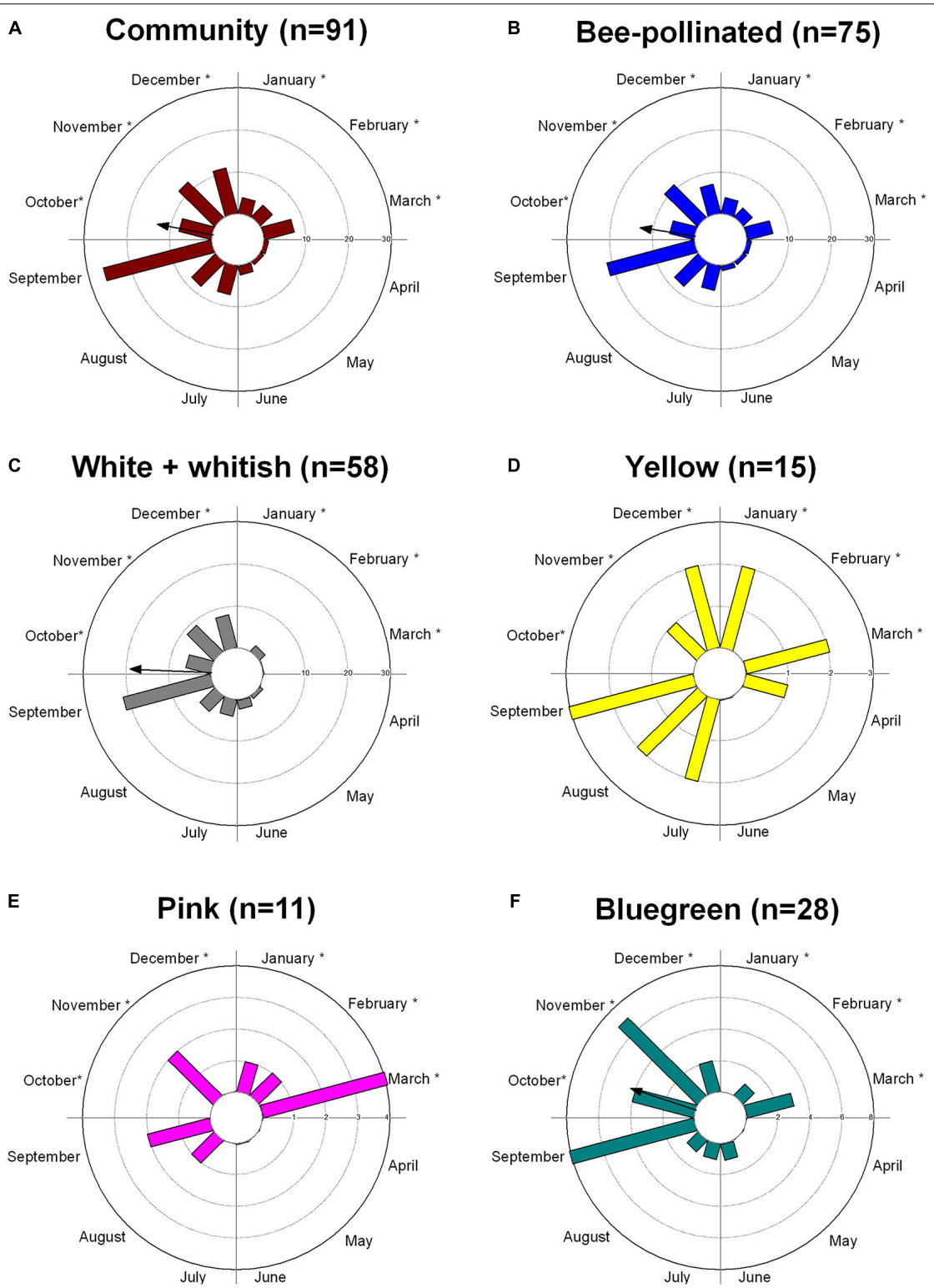


FIGURE 2 | Flowering patterns of a woody cerrado vegetation (Itirapina, southeastern Brazil) based on the number of species presenting flowering peak in each month: **(A)** for the cerrado community (91 species), **(B)** for 75 species pollinated by bees, **(C–E)** by flower color according to the human-color vision, and **(F)** for the bee-bluegreen flowers, the predominant bee color in the community. The arrows point to the significant mean angles or dates, and the arrows' length corresponds to the *r* vector value (0–1). Arrows are not presented for bimodal patterns **(D,E)**. The rainy warm season occurs from October to March, represented by an *, and the dry cooler season from April to September; a transitional dry-to-wet season is observed between September and October.

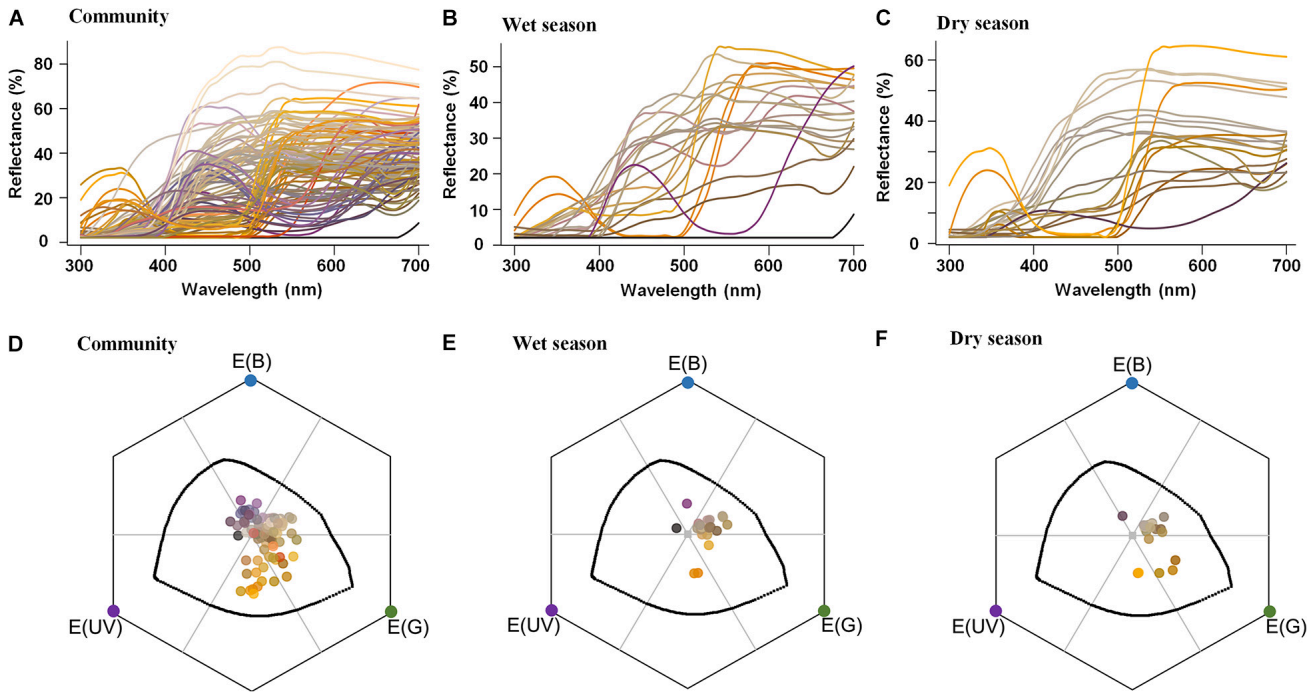


FIGURE 3 | Reflectance spectra **(A–C)** and color loci distribution **(D–F)** in the bee visual space of flowers from 99 animal-pollinated cerrado species **(A,D)** and of flowers from 22 bee-pollinated species flowering mainly during the wet season **(B,E)** and 21 bee-pollinated species flowering mainly during the dry season **(C,F)**. Each line in the reflectance spectra **(A–C)** and each point in the hexagons **(D–F)** are represented by the correspondent human color according to the function *spec2rgb* for R (Maia et al., 2018). In the bee visual space, represented by hexagons **(D–F)**, the gray point represents the achromatic center (color locus of the leaf background), and the colorful points in the vertices represent each bee-photoreceptor: blue E (B), green E (G), and ultraviolet E (UV). The black line inside the hexagon delimits the maximum sensitivity of each photoreceptor to a monochromatic light. Each of the six parts of the hexagon, limited by a gray line, represents a bee-color category—clockwise direction from E (B): blue, bluegreen, green, UVgreen, UV, and UVblue.

species with bee-bluegreen flowers peaked in the dry season and 64% in the wet season (**Figure 3** and **Supplementary Table S1**). Conversely, the percentage of species with bee-green flowers peaking in the dry season was higher (14%) than that in the wet season (4.5%), whereas 18% of species with bee-blue flowers peaked in the wet season, and only 5% (one species) in the dry season (5%) (**Figure 3** and **Supplementary Table S1**).

Floral contrasts did not differ between seasons: color contrast ($W = 212$, $p = 0.65$) and green contrast ($W = 235$, $p = 0.93$) (**Figures 4A,B**). Additionally, flowers of both seasons showed higher values of color contrast against the background during the dry season (dry season flowers: $V = 225$, $p < 0.001$; wet season flowers: $V = 6$, $p < 0.001$) and higher values of green contrast against the background during the wet season (dry season flowers: $V = 0$, $p < 0.001$; wet season flowers: $V = 232$, $p < 0.001$).

DISCUSSION

The plant species in the cerrado community studied flowered throughout the year, where the highest diversity of flowering species and flower colors was observed in September, at the end of the dry season. Bee pollination was the dominant pollination system with bee-pollinated flowers available all year long, decreasing in quantity only during the early dry season (April to June). White and bee-bluegreen are the most common flower colors according to human and bee visual systems, respectively. Flowers produced during the dry and wet seasons showed similar contrasts against their corresponding background. However, regardless of the flowering season, the background of the dry season promoted the highest color contrasts, whereas the green contrast was higher against the wet season background.

Bees and Flower Color Patterns

Based on the human visual system, white, yellow, and pink were the most common flower colors, similar to what has been described for other cerrado areas (Oliveira and Gibbs, 2000, 2002; Gottsberger and Silberbauer-Gottsberger, 2006). Similarly, bees were responsible for the pollination of more than 70% of species in our community and at least 50% in other cerrado communities (Oliveira and Gibbs, 2000, 2002; Gottsberger and Silberbauer-Gottsberger, 2006). September was the month with the highest flowering intensity coupled with the highest occurrence of bees (D'Avila and Marchini, 2008). Bee activity coincides to the number of flowering species and resource availability: a greater number of bee species have been observed from August to September and from November to

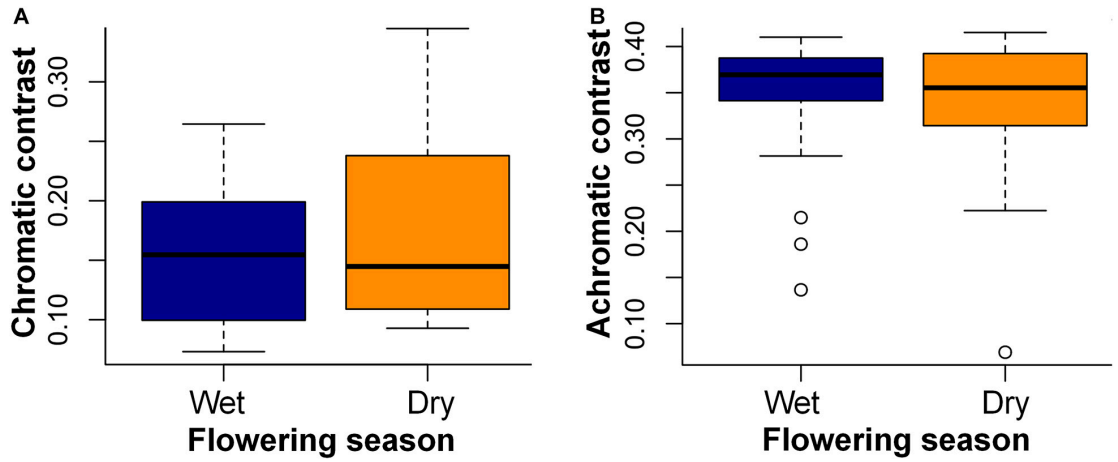


FIGURE 4 | Main flowering season and flower contrasts against the leaf background calculated for 43 bee-pollinated species sampled in the study site. **(A)** Color contrast and **(B)** green contrast against the leaf background of the dry and wet seasons. Horizontal lines indicate the mean value of contrasts, and vertical lines indicate the standard deviation. Chromatic contrasts are given in hexagon units and correspond to the Euclidian distance between a flower color locus and the center of the bee hexagon; the green contrasts are stimulus of the green photoreceptor adapted to the background (Chittka, 1992).

June (Gottsberger and Silberbauer-Gottsberger, 2006; D'Avila and Marchini, 2008).

The high flowering intensity and the high number of species reaching the flowering peak at the end of the dry season corroborate the pattern observed for other cerrado communities (Oliveira and Gibbs, 2000). Even though precipitation, temperature, and photoperiod are triggers that induce flowering of cerrado species (Batalha et al., 1997; Oliveira, 2008; Pirani et al., 2009), cerrado woody species are less dependent on seasonal restrictions, and water availability is not a limiting factor for flowering (Batalha et al., 1997; Pirani et al., 2009). Moreover, resource allocation, pollinator competition, phylogenetic restrictions, optimal dispersion period, and occasional fire events are also important factors that can affect flowering (Miranda et al., 1998; Oliveira, 2008; Escobar et al., 2018).

Flowers of all colors peaked at the end of the seasons, considered a transition between seasons (Escobar et al., 2018). The predominant white and yellow flowers peaked at the end of the dry season, whereas pink flowers peaked at the end of the wet season. The pink flower pattern may be related to the predominance of wind dispersal among these species, in which seeds must be dispersed during the dry season (Batalha and Martins, 2004; Escobar et al., 2018). In seasonal forests, the dry season has long been hypothesized to favor pollination by insects due to high light availability and low precipitation (Janzen, 1967). In the cerrado, eusocial Apidae bees are less susceptible to low temperatures and low relative humidity and are considered active pollinators of cerrado plant species all year long (Almeida and Laroca, 1988; D'Avila and Marchini, 2008; Abrahamczyk et al., 2011). Considering the importance of *A. mellifera* and *T. spinipes* as pollinators in cerrado communities (D'Avila and Marchini, 2008), yellow and white flowers are definitely important food sources during the dry season, maintaining pollinator activity all year long (Ramírez, 2006). In addition, by flowering during the dry season, these species avoid competition, decreasing the interference of other flowering plants (Ramírez, 2006).

Species with bee-bluegreen flowers peaked mainly at the end of the dry season (September), but flowering was observed throughout most of the year, confirming the importance of these flowers as food sources for different pollinators. According to the trichromatic vision of bees, bee-bluegreen was the most common flower color in the cerrado. This bee color includes the bee-pollinated flowers perceived by humans as white or whitish (Kevan et al., 1996), corroborating the importance of bees as pollinators in the cerrado. In addition, the bee-bluegreen color also includes pale colors related to many other groups of pollinators, such as moths, flies, beetles, and bats (Gottsberger and Silberbauer-Gottsberger, 2006). Even though information regarding UV reflectance is lacking, we know, for example, that the bee-UVgreen and bee-green flowers correspond to human-yellow flowers (Kevan et al., 1996), which were produced throughout the year, and that the bee-blue and bee-UVblue flowers correspond mainly to the human-pink flowers (Kevan et al., 1996), which were more concentrated in the wet season.

Seasonality and Visual Contrasts

Flower color contrasts were affected by abiotic conditions as the background coloration was significantly different between the dry and wet seasons (Camargo et al., 2014), suggesting that it acts as a selective pressure for flower color and detection. Foraging in complex backgrounds are challenging to bees, as fluctuations in the perceived contrasts are frequent when compared with a homogeneous achromatic background (Telles et al., 2017).

Even though flower color phenology differed, flower contrasts were similar between seasons. Plants flowering in the wet and dry seasons were equally conspicuous against their corresponding background. However, we found that the background coloration affected flower contrasts regardless of flowering season. The

leaf senescence peak in our community occurs in the dry season, promoting a yellowish background (Alberton et al., 2014; Camargo et al., 2018) and thus higher color contrast, which most likely maximizes flower detection in a period under less favorable environmental conditions and of reduced flower activity (Bukovac et al., 2017b; Telles et al., 2017). Most cerrado plants produce new leaves at the end of the dry season, with a predominance of greener leaves throughout the wet season (Alberton et al., 2014; Camargo et al., 2018). The higher green contrast against the background of the wet season may be important to guarantee flower detection against new and mature green leaves. Hence, the different color contrasts are favored according to their importance for flower detection, ensuring flower conspicuousness across seasons. It is worth highlighting that during the transitions between seasons, the background coloration is also changing and can vary from a more yellowish to green (or greener to yellow) according to variations in the length of the seasons and associated leafing patterns over the years (Alberton et al., 2014; Camargo et al., 2018). Therefore, in seasonally dry ecosystems, where flowering peaks mainly at the end of the seasons (Morellato et al., 2013), it is also advantageous to produce flowers that contrast with the leaf coloration of both seasons.

We evaluated 11 years of phenology data to show, for the first time, that seasonality affects flower color diversity and flower contrasts in seasonal ecosystems, such as the cerrado. Distinctions in the peak of certain flower colors along with the maintenance of color diversity over the year may favor the presence and diversity of bees and other pollinators throughout the year in the community (Ramírez, 2006; Genini, 2011). We have demonstrated the importance of considering the bee-color vision associated to the natural background and its seasonal changes when analyzing flower color contrasts. Background coloration influenced color contrasts, favoring flower conspicuousness to bees according to seasons. When linking flower color, seasonality, and bee pollination, we found that temporal patterns of flower colors are likely adapted to abiotic and biotic constraints, such as climate seasonality, bee activity, visual system, and behavior. Thus, our results provide new insights for future research regarding the temporal patterns of plant–pollination interactions in seasonally dry ecosystems.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AEM, MGGC and LPCM conceived and designed the study. MGGC and AEM conducted the field work and analyzed the data. AEM wrote the original draft of the manuscript. LPCM provided financial support and management. All authors contributed to revisions and editions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.594538/full#supplementary-material

Supplementary Figure 1 | (A,B) Location of the cerrado study site at Itirapina, São Paulo State, Brazil (above with a yellow pin) and the study site in detail (below), where the four red lines, along which the 36 transects were distributed, represent the general location of plots where phenology species were sampled and are observed in the long-term cerrado phenology monitoring [map data: **(A)** Google, Image Landsat/Copernicus; **(B)** Google, Maxar Technologies]; **(C)** general view of the cerrado *sensu stricto* vegetation (photo: AEM).

Supplementary Figure 2 | Flowering patterns of a woody cerrado vegetation (Itirapina, southeastern Brazil) based on the average monthly intensity. **(A)** For the cerrado community (91 species), **(B)** for 75 species pollinated by bees, **(C–E)** by flower color according to the human-color vision. The rainy warm season occurs from October to March, represented by an *, and the dry cooler season from April to September; a transitional dry-to-wet season is observed between September and October.

Supplementary Figure 3 | Flowering patterns of a woody cerrado vegetation (Itirapina, southeastern Brazil) based on the number of species presenting flowering peak in each month, for 49 species with phenological and reflectance data. The rainy warm season occurs from October to March, represented by an *, and the dry cooler season from April to September; a transitional dry-to-wet season is observed between September and October.

REFERENCES

Abrahamczyk, S., Kluge, J., Gareca, Y., Reichle, S., and Kessler, M. (2011). The influence of climatic seasonality on the diversity of different tropical pollinator groups. *PLoS One* 6:e27115. doi: 10.1371/journal.pone.0027115

Alberton, B., Almeida, J., Helm, R., Torres, R., da, S., Menzel, A., et al. (2014). Using phenological cameras to track the green up in a cerrado savanna and its on-the-ground validation. *Ecol. Inform.* 19, 62–70. doi: 10.1016/j.ecoinf.2013.12.011

Alberton, B., Torres, R., da, S., Silva, T. S. F., da Rocha, H. R., Moura, M. S. B., et al. (2019). Leafing patterns and drivers across seasonally dry tropical communities. *Remote Sens.* 11:2267. doi: 10.3390/rs11192267

Almeida, M. C., and Laroca, S. (1988). *Trigona spinipes* (Apidae, Meliponinae): taxonomia, bionomia e relações tróficas em áreas restritas. *Acta Biológica Paranaense* 17, 67–108.

Augspurger, C. K. (1981). Reproductive Synchrony of a Tropical Shrub: experimental studies on effects of pollinators and seed predators in *Hybanthus prunifolius* (Violaceae). *Ecology* 62, 775–788. doi: 10.2307/1937745

Augspurger, C. K. (1983). Phenology, flowering synchrony, and fruit set of six neotropical shrubs. *Biotropica* 15:257. doi: 10.2307/2387650

Batalha, M. A., Aragaki, S., and Mantovani, W. (1997). Phenological variations of the cerrado species in Emas-Pirassununga, SP. *Acta Bot. Brasilica* 11, 61–78. doi: 10.1590/S0102-33061997000100007

Batalha, M. A., and Martins, F. R. (2004). Reproductive phenology of the cerrado plant community in Emas National Park (central Brazil). *Aust. J. Bot.* 52, 149–161. doi: 10.1071/BT03098

Binkenstein, J., and Schaefer, H. M. (2015). Flower colours in temperate forest and grassland habitats: a comparative study. *Arthropod. Plant. Interact.* 9, 289–299. doi: 10.1007/s11829-015-9369-9

Bukovac, Z., Dorin, A., Finke, V., Shrestha, M., Garcia, J., Avarguès-Weber, A., et al. (2017a). Assessing the ecological significance of bee visual detection and colour discrimination on the evolution of flower colours. *Evol. Ecol.* 31, 153–172. doi: 10.1007/s10682-016-9843-6

Bukovac, Z., Shrestha, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017b). Why background colour matters to bees and flowers. *J. Comp. Physiol. A Neuroethol. Sens. Neural, Behav. Physiol.* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Camargo, M. G. G., Cazetta, E., Morellato, L. P. C., and Schaefer, H. M. (2014). Characterizing background heterogeneity in visual communication. *Basic Appl. Ecol.* 15, 326–335. doi: 10.1016/j.baae.2014.06.002

Camargo, M. G. G., Cazetta, E., Schaefer, H. M., and Morellato, L. P. C. (2013). Fruit color and contrast in seasonal habitats-a case study from a cerrado savanna. *Oikos* 122, 1335–1342. doi: 10.1111/j.1600-0706.2013.00328.x

Camargo, M. G. G., de Carvalho, G. H., Alberton, B., de, C., Reys, P., and Morellato, L. P. C. (2018). Leafing patterns and leaf exchange strategies of a cerrado woody community. *Biotropica* 50, 442–454. doi: 10.1111/btp.12552

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181.

Chittka, L., Shmida, A. V. I., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Chittka, L., Thomson, J. D., and Waser, N. M. (1999). Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86, 361–377. doi: 10.1007/s001140050636

Cortés-Flores, J., Hernández-Esquivel, K., González-Rodríguez, A., and Ibarra-Manríquez, G. (2017). Flowering phenology, growth forms, and pollination syndromes in tropical dry forest species: influence of phylogeny and abiotic factors. *Am. J. Bot.* 104, 39–49. doi: 10.3732/ajb.1600305

Coutinho, L. M. (2006). O conceito de bioma. *Acta Botânica Bras.* 20, 13–23. doi: 10.1590/S0102-33062006000100002

Dalrymple, R. L., Hui, F. K. C., Flores-Moreno, H., Kemp, D. J., and Moles, A. T. (2015). Roses are red, violets are blue-so how much replication should you do? An assessment of variation in the colour of flowers and birds. *Biol. J. Linn. Soc.* 114, 69–81. doi: 10.1111/bij.12402

D'Avila, M., and Marchini, L. C. (2008). Faunistic analysis of flower-visiting Hymenoptera in cerradão fragment in Itirapina, SP. *Ciência Florestal* 18, 271–279. doi: 10.5902/19805098465

Dyer, A. G., Boyd-Gerny, S., Mcloughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B Biol. Sci.* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., Boyd-Gerny, S., Shrestha, M., Lunau, K., Garcia, J. E., Koethe, S., et al. (2016). Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 202, 603–613. doi: 10.1007/s00359-016-1101-4

Dyer, A. G., Whitney, H. M., Arnold, S. E. J., Glover, B. J., and Chittka, L. (2006). Bees associate warmth with floral colour. *Nature* 442:525. doi: 10.1038/442525a

Escobar, D. F. E., Silveira, F. A. O., and Morellato, L. P. C. (2018). Timing of seed dispersal and seed dormancy in Brazilian savanna: two solutions to face seasonality. *Ann. Bot.* 121, 1197–1209. doi: 10.1093/aob/mcy006

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Fournier, L. A. (1974). Un método cuantitativo para la medición de características fenológicas em árboles. *Turrialba* 24, 422–423.

Frankie, G. W., Baker, H. G., and Opler, P. A. (1974). Comparative phenological studies of trees in tropical wet and dry forests in the Lowlands of Costa Rica. *J. Ecol.* 62:881. doi: 10.2307/2258961

Genini, J. (2011). *Sazonalidae e as redes de interações planta-polinizador*. Ph.D. thesis, São Paulo State University, Rio Claro, SP

Gentry, A. H. (1974). Flowering phenology and diversity in tropical Bignoniaceae. *Biotropica* 6, 64–68. doi: 10.2307/2989698

Gottsberger, G., and Silberbauer-Gottsberger, I. (2006). *Life in the Cerrado: Pollination and Seed Dispersal*, Ulm: Reta Verlag.

Janzen, D. H. (1967). Why mountain passes are higher in the tropics author. *Am. Nat.* 101, 233–249. doi: 10.1086/282487

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1, 252–284. doi: 10.1016/1360-1385(96)20008-1

Klink, C. A., and Machado, R. B. (2005). A conservação do Cerrado brasileiro. *Megadiversidade* 1, 147–155. doi: 10.1590/S0100-69912009000400001

Kovach, W. L. (2011). *Oriana: Circular Statistics for Windows (Version 4.0)*. Pentraeth: Kovach Computer Services.

Lieth, H. (1974). "Introduction to phenology and modeling of seasonality," in *Phenology and Seasonality Modeling*, ed. H. Lieth (Springer Verlag: Berlin), 3–19.

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19. doi: 10.1007/BF00243394

Maia, R., White, T., Gruson, H., Eliason, C., and Bitton, P. (2018). *Perceptual Analysis, Visualization and Organization of Spectral Color Data in R. Version 2.0*.

Martins, A. E. (2019). *Flower Color Patterns and Pollination in Seasonal Vegetations*. Ph.D. thesis, São Paulo State University, Rio Claro, SP.

Menzel, R., and Backhaus, W. (1991). "Colour vision in insects." in *Vision and Visual Dysfunction: The Perception of Colour*, ed P. Gouras (London: Pan MacMillan), 262–288.

Miranda, H. S., Bustamante, M. M. C., and Miranda, A. C. (1998). "The Fire Factor," in *The Cerrados of Brazil: Ecology and Natural History of a Neotropical Savanna*, eds P. S. Oliveira and R. J. Marquis (New York, NY: Columbia University Press), 51–68. doi: 10.7312/oliv12042

Morellato, L. P. C., Alberton, B., Alvarado, S. T., Borges, B., Buisson, E., Camargo, M. G. G., et al. (2016). Linking plant phenology to conservation biology. *Biol. Conserv.* 195, 60–72. doi: 10.1016/j.biocon.2015.12.033

Morellato, L. P. C., Camargo, M. G. G., and Gressler, E. (2013). "A Review of Plant Phenology in South and Central America," in *Phenology: An Integrative Environmental Science*, ed. M. D. Schwartz (Dordrecht: Springer), 1–610. doi: 10.1007/978-94-007-6925-0

Morellato, P. C., and Leitao-Filho, H. F. (1996). Reproductive phenology of climbers in a Southeastern Brazilian Forest. *Biotropica* 28, 180–191. doi: 10.2307/2389073

Morellato, P. L. C., Alberti, L. F., and Hudson, I. H. (2010). "Applications of circular statistics in plant phenology: a case studies approach" in *Phenological Research: Methods for Environmental and Climate Change Analyses*, ed. I. L. Hudson, and M. R. Keatley (Springer, Dordrecht) 339–360. doi: 10.1007/978-90-481-3335-2.

Morellato, P. L. C., Talora, D. C., Takahasi, A., Bencke, C. C., Romera, E. C., and Zipparro, V. B. (2000). Phenology of Atlantic rain forest trees: a comparative study. *Biotropica* 32:811. doi: 10.1646/0006-3606(2000)032[0811:poarft]2.0.co;2

Newstrom, L. E., Frankie, G. W., and Baker, H. G. (1994). A new classification for plant phenology based on flowering patterns in Lowland tropical rain forest trees at La Selva. *Costa Rica. Biotropica* 26:141. doi: 10.2307/2388804

Oliveira, P. E. (2008). "Fenologia e Biologia reprodutiva das espécies de Cerrado," in *Ecologia e Flora*, eds S. M. Sano, S. P. Almeida, and J. F. Ribeiro (Brasília: Embrapa Cerrados).

Oliveira, P. E., and Gibbs, P. E. (2000). Reproductive biology of woody plants in a cerrado community of Central Brazil. *Flora* 195, 311–329. doi: 10.1016/S0367-2530(17)30990-8

Oliveira, P. E., and Gibbs, P. E. (2002). "Pollination and reproductive biology in cerrado plant communities," in *The Cerrados of Brazil: Ecology and Natural History of a Neotropical Savanna*, eds P. E. Oliveira and R. J. Marquis (New York, NY: Columbia University), 329–347. doi: 10.7312/oliv12042-016

Ollerton, J. (2017). Pollinator diversity: distribution. ecological function, and conservation. *Annu. Rev. Ecol. Evol. Syst.* 48, 353–376. doi: 10.1146/annurev-ecolsys-110316-022919

Opler, P. A., Frankie, G. W., and Baker, H. G. (1980). Comparative phenological studies of treelet and shrub species in tropical wet and dry forest in the lowlands of Costa Rica. *J. Ecol.* 68, 167–188. doi: 10.2307/2259250

Pirani, F. R., Sanchez, M., and Pedroni, F. (2009). Phenology of a tree community in a cerrado sensu stricto. Barra do Garças, Mato Grosso state, Brasil. *Acta Bot. Brasilica* 23, 1096–1109. doi: 10.1590/s0102-33062009000400019

Ramírez, N. (2006). Temporal variation of pollination classes in a tropical Venezuelan plain: the importance of habitats and life forms. *Can. J. Bot.* 84, 443–452. doi: 10.1139/b06-015

Reys, P., Camargo, M. G. G. D., Grombone-Guaratini, M. T., Teixeira, A. D. P., Assis, M. A., and Morellato, L. P. C. (2013). Structure and floristic composition of a Cerrado sensu stricto and its relevance to ecological restauration. *Hoehnea* 40, 449–464.

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Smith-Ramírez, C., Armesto, J. J., and Figueroa, J. (1998). Flowering, fruiting and seed germination in Chilean rain forest myrtaceae: ecological and phylogenetic constraints. *Plant Ecol.* 136, 119–131. doi: 10.1023/A:1009730810655

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Telles, F. J., Corcobado, G., Trillo, A., and Rodríguez-Gironés, M. A. (2017). Multimodal cues provide redundant information for bumblebees when the stimulus is visually salient, but facilitate red target detection in a naturalistic background. *PLoS One* 12:e0184760. doi: 10.1371/journal.pone.0184760

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

Vogado, N. O., de Camargo, M. G. G., Locosselli, G. M., and Morellato, L. P. C. (2016). Edge effects on the phenology of the Guamirim. *Myrcia guianensis* (Myrtaceae), a cerrado tree, Brazil. *Trop. Conserv. Sci.* 9, 291–312. doi: 10.1177/194008291600900115

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Changes at a Critical Branchpoint in the Anthocyanin Biosynthetic Pathway Underlie the Blue to Orange Flower Color Transition in *Lysimachia arvensis*

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Anthocyanins are the primary pigments contributing to the variety of flower colors among angiosperms and are considered essential for survival and reproduction. Anthocyanins are members of the flavonoids, a broader class of secondary metabolites, of which there are numerous structural genes and regulators thereof. In western European populations of *Lysimachia arvensis*, there are blue- and orange-petaled individuals. The proportion of blue-flowered plants increases with temperature and daylength yet decreases with precipitation. Here, we performed a transcriptome analysis to characterize the coding sequences of a large group of flavonoid biosynthetic genes, examine their expression and compare our results to flavonoid biochemical analysis for blue and orange petals. Among a set of 140 structural and regulatory genes broadly representing the flavonoid biosynthetic pathway, we found 39 genes with significant differential expression including some that have previously been reported to be involved in similar flower color transitions. In particular, *F3′5′H* and *DFR,* two genes at a critical branchpoint in the ABP for determining flower color, showed differential expression. The expression results were complemented by careful examination of the SNPs that differentiate the two color types for these two critical genes. The decreased expression of *F3′5′H* in orange petals and differential expression of two distinct copies of *DFR*, which also exhibit amino acid changes in the color-determining substrate specificity region, strongly correlate with the blue to orange transition. Our biochemical analysis was consistent with the transcriptome data indicating that the shift from blue to orange petals is caused by a change from primarily malvidin to largely pelargonidin forms of anthocyanins. Overall, we have identified several flavonoid biosynthetic pathway loci

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likely involved in the shift in flower color in *L. arvensis* and even more loci that may represent the complex network of genetic and physiological consequences of this flower color polymorphism.

Keywords: *DFR*, *F3′5′H,* flavonoid biosynthetic pathway, malvidin, pelargonidin, RNA-Seq

INTRODUCTION

Flower color plays a fundamental role in pollinator attraction and plant reproduction (Fenster et al., 2004). Changes in flower color may produce pollinator shifts leading to speciation (Rausher, 2008; Ortiz-Barrientos, 2013; Van der Niet et al., 2014). Abiotic factors have received far less attention, but they may also impose selection on flower color (e.g., temperature, precipitation, solar radiation; Strauss and Whittall, 2006; Rausher, 2008; Van der Kooi et al., 2019; Dalrymple et al., 2020; Koski et al., 2020). The palette of flower color is diverse and there are a variety of mechanisms to control flower colors (cell shape, co-pigments, pH, etc.). However, large differences in flower color are mainly controlled by the underlying pigments such as chlorophylls, carotenoids, betalains and flavonoids (Tanaka et al., 2008). Anthocyanins are the most common group of flavonoid pigments present in flowers, and differences in the number of hydroxyl groups on their B-ring characterize the three main types: pelargonidin-derived anthocyanins (orange or red; one hydroxyl group), cyanidin-derived anthocyanins (magenta; two hydroxyl groups) and delphinidin-derived anthocyanins (including malvidin conferring blue or purple; three hydroxyl groups; Davies, 2009; Wessinger and Rausher, 2012, 2013; Ng et al., 2018).

Flower color variation exists both between and within species across angiosperms. Flower color transitions are common between related species (Zufall and Rausher, 2004; Tripp and Manos, 2008; Streisfeld and Rausher, 2009; Smith and Rausher, 2011; Wessinger and Rausher, 2013; Roberts and Roalson, 2017). However, some lineages exhibit striking flower color variation within species (Gigord et al., 2001; Schemske and Bierzychudek, 2007; Gómez et al., 2020). Flower color polymorphisms, the coexistence of two or more discrete flower color phenotypes within a population (Huxley, 1955; Schemske and Bierzychudek, 2007), is relatively common and concentrated in some families of angiosperms (Narbona et al., 2018). Flower color polymorphisms are frequently caused by the loss of anthocyanins (i.e., polymorphisms usually involving white-flowered plants) as in the cases of *Ipomoea purpurea* (purple and white; Rausher and Simms, 1989), *Linanthus parryae* (blue and white; Schemske and Bierzychudek, 2007), *Ruellia simplex* (purple, pink, and white; Freyre et al., 2015), *Parrya nudicaulis* (purple and white; Dick et al., 2011), *Raphanus sativus* (pink, bronze, yellow and white; Irwin and Strauss, 2005) and *Primula vulgaris* (purple, blue, pink, white, and yellow; Shipunov et al., 2011). Less commonly, flower color polymorphisms are caused by changes in anthocyanin composition as in *Ipomoea nil* (blue, magenta, purple, and red; Morita et al., 2005), *Phlox drummondii* (blue and red; Hopkins and Rausher, 2011) and *Lysimachia arvensis* (blue and orange; Arista et al., 2013). Streisfeld and Rausher (2011) predicted that evolutionary transitions in floral color are more likely caused by regulatory mutations in ABP genes instead of mutations in the coding sequence (CDS), because regulatory changes can be tissue-specific and are thus less likely to have deleterious pleiotropic effects. In this study, we focus on the flavonoid genes and compounds underlying the blue and orange flower color variation of *Lysimachia arvensis*.

Anthocyanins are synthesized by the anthocyanin biosynthetic pathway (ABP) – a highly conserved pathway among angiosperms and one of the best studied pathways for the production of secondary metabolites in plants (Holton and Cornish, 1995; Davies and Schwinn, 2006; Ho and Smith, 2016; Wheeler and Smith, 2019). The ABP is part of the flavonoid biosynthetic pathway, a broader metabolic pathway emerging from phenylpropanoid metabolism that includes flavone, flavonol, and anthocyanin synthesis plus the post-translational modifying steps like acylation and glycosylation (Clegg and Durbin, 2000; Sobel and Streisfeld, 2013; Chen et al., 2018; Gao et al., 2019). Approximately 100 structural genes (KEGG pathway database[1]) are involved in this broad flavonoid biosynthetic pathway. In addition, tissue-specific transcription factors play an important role in this pathway (Quattrocchio et al., 2006; Zhao et al., 2013), regulating those structural genes through the MYB-bHLH-WDR complex (MBW; Xu et al., 2015; Gao et al., 2019). This regulatory complex is conserved among vascular plants; however, the structural genes that they regulate varies among angiosperm lineages (Hichri et al., 2011; Lai et al., 2013; Albert et al., 2014; Xu et al., 2015). Regulatory complexity (e.g., tissue-specific regulation) is conferred by the large gene families for these proteins (e.g., R2R3 MYBs; Stracke et al., 2001; WD40; Xu et al., 2015). In particular, MYB regulators tend to be disproportionately involved in floral anthocyanin variation both within and between species.

The number of studies describing the causes of flower color variation in the wild is rapidly growing (e.g., Dick et al., 2011; Yuan et al., 2013; Casimiro-Soriguer et al., 2016; Kellenberger et al., 2019). Wheeler and Smith (2019) reviewed 15 studies involving natural evolutionary transitions between anthocyanin pigment types in which all 15 examples involved decreased expression in either *F3′H* or *F3′5′H*, or both genes (Wessinger and Rausher, 2012; Wheeler and Smith, 2019). Furthermore, two of these cases involved *DFR* mutations conferring substrate specificity, a secondary modification to redirect the flux directly down the pelargonidin branch of the ABP (Johnson et al., 2001; Zufall and Rausher, 2004; Smith and Rausher, 2011; Wheeler and Smith, 2019). According to Streisfeld and Rausher (2011), flower color variation associated with anthocyanin loss is more likely to be caused by regulatory genes mutations to

[1]https://www.genome.jp/kegg/pathway.html

avoid deleterious pleiotropic effects, while flower color variation associated with changes in anthocyanin type are more likely to be caused by mutations in structural genes, however Wheeler and Smith's (2019) findings suggest both regulatory and structural changes are often correlated with the blue to red transition in natural flower color changes. Traditional approaches to finding the genes underlying flower color variation involve sequencing and determining expression in a relatively narrow group of candidate genes in the ABP (Le Maitre et al., 2019) which we will refer to as the narrow ABP. Alternatively, transcriptomic approaches using RNA-Seq allow a much broader investigation of the underlying causes of flower color variation (e.g., across the broader flavonoid biosynthetic pathway). For non-model species, *de novo* transcriptome assembly can be used to estimate genes with significant differential expression (DEGs; Wang et al., 2009; Martin and Wang, 2011; Grabherr et al., 2011; Anders et al., 2013; Haas et al., 2013; Butler et al., 2014; Casimiro-Soriguer et al., 2016; Chen et al., 2018; Duan et al., 2020). Even using a transcriptome approach, most studies of anthocyanin loss have focused on a narrow group of ABP candidate genes (Lulin et al., 2012; Lou et al., 2014; Casimiro-Soriguer et al., 2016; Gao et al., 2016; Lang et al., 2019; Li L. et al., 2019; Duan et al., 2020; Zhang et al., 2020). Herein, we describe the *de novo* assembled transcriptome for blue- and orange-flowered *Lysimachia arvensis* to determine differentially expressed genes among the broader flavonoid biosynthetic pathway.

Lysimachia arvensis (L.) Manns & Anderb. (Primulaceae), formerly *Anagallis arvensis*, is a self-incompatible (Gibbs and Talavera, 2001), tetraploid (2n = 40; Pujadas, 1997; Moneim et al., 2003) annual species displaying a petal color polymorphism. Blue- and orange-flowered plants, traditionally described as blue- and red-flowered plants (Ferguson, 1972), occur in monomorphic and polymorphic populations across its native range in Europe (Arista et al., 2013; Ortiz et al., 2015). The blue petals of *L. arvensis* contain malvidin derivatives, whereas orange petals contain pelargonidin derivatives (Lawrence et al., 1939; Harborne, 1968; Ishikura, 1981). Although both *L. arvensis* flower colors have long been described as different morphs, recent molecular phylogenetic studies indicate that these flower color phenotypes reflect very closely related, yet distinct, evolutionary lineages (Jiménez-López, 2019; Jiménez-López et al., in review) with some degree of reproductive isolation (Jiménez-López et al., 2020). Pollinators (mainly *Halictus* spp. bees; Ortiz et al., 2015; Jiménez-López et al., 2019) exhibit a preference for the blue individuals when given a choice. Across Europe, there is a latitudinal cline in flower color from north to south. Completely orange populations are common in the more mesic temperate latitudes of northern and central Europe whereas completely blue populations are most common in the more xeric Mediterranean environments of southern Europe with polymorphic populations in between (Arista et al., 2013). In combination with the ecological divergence of these two flower color variants across the species range, this case of incipient speciation provides an opportunity to investigate the role of the flavonoid biosynthetic pathway during speciation (Coyne and Orr, 1998; Widmer et al., 2009; Reiseberg and Blackman, 2010).

In this study, we aim to identify the underlying genetic and biochemical causes of flower color variation in *L. arvensis*. Previous studies have found that gene expression and/or gene structure at a critical branch-point in the anthocyanin biosynthetic pathway involving two genes, *F3′5′H* and *DFR,* are involved in most blue to red/orange flower color shifts (malvidin/cyanidin to pelargonidin; Davies, 2009; Streisfeld and Rausher, 2009, 2011; Smith et al., 2012; Ho and Smith, 2016; Wheeler and Smith, 2019). In order to identify the underlying genetic causes of petal color polymorphism in *L. arvensis*, we performed *de novo* transcriptome assembly from RNA-Seq data. We then tested for differential expression across a broad panel of 94 structural and 46 regulatory genes involved in the flavonoid biosynthetic pathway. We also examined these two candidate genes for non-synonymous single nucleotide polymorphisms (SNPs) fixed between blue and orange flowers in reference to the known functional domains as described for other species (Johnson et al., 2001; Seitz et al., 2007; Smith et al., 2012; Guo et al., 2019). Finally, we interpret our transcriptome results in light of the flavonoid biochemical profiles of both flower colors using Ultra-High Performance Liquid Chromatography (UHPLC-MS).

MATERIALS AND METHODS

Plant Material

For transcriptomic analysis, we collected seeds from blue-flowered plants that belong to a primarily blue-flowered population (blue:orange ratio = 10:1; Spain, Andalusia, Hinojos; 37°17′37″ N, 6°25′15″ W) and orange-flowered plants that belong to a monomorphic orange population (France, Corsica, Solenzara; 41°51′19″ N, 9°21′16″ E). Voucher specimens were deposited in the University of Seville Herbarium (Herbarium Numbers: SEV286467 and SEV279123). Although there are benefits from studying a mixed population in order to control for differences in genetic background that can arise due to geographic separation and/or local adaptation in allopatry, we used these two populations because we previously obtained four generations of inbred lines that exhibit reduced heterozygosity, which facilitates transcriptome assembly and analysis. These seeds were sown in a glasshouse at Santa Clara University (CA, United States), where the RNA extractions of blue- and orange-flowered samples were performed on the same days, alternating the preparation of samples between flower colors, in relatively homogeneous environmental conditions. We sampled all five petals from ∼30 flowers per plant, from eight blue- and eight orange-flowered plants. We avoided the "bullseye" at the base of each petal because this study is focused on the blue and orange flower color polymorphism, and we have no evidence that the bullseye is different between morphs based on visible and UV digital images (**Supplementary Figure 1**). All samples were taken from first-day anthesis flowers. Immediately after excision, petals were placed in cryotubes that were immediately immersed in liquid nitrogen and stored at −80°C to avoid subsequent changes in gene expression and RNA degradation. For flavonoid profiling, we sampled one blue and one orange plant from Spain

and confirmed with 13 blue and 10 orange glasshouse-grown introduced plants from California. Each sample was composed of between 10 and 15 petals without the central bullseye. Petal samples were stored in microtubes containing 200 μl of MeOH with 1% HCl.

RNA Extraction and *de novo* Assembly

Petals were homogenized using a mortar and pestle. Total RNA was then extracted following the Qiagen RNeasy® Plant Mini Kit protocol (Qiagen, Germany) with the addition of PEG 20,000 mol. wt. (550 μl, 2%; Gehrig et al., 2000) before the first filtering step. The addition of PEG was essential to achieve reasonable RNA concentrations for library preparation and sequencing. RNA samples were stored at −80°C until further analysis. RNA concentration and purity was initially assessed with a Nanodrop Nd-1000 (Thermo Fisher Scientific) and agarose gel, and then confirmed with a Bioanalyzer (Agilent, Santa Clara, CA, United States) before sequencing.

Individual libraries were barcoded, multiplexed and sequenced as 150 bp paired-end reads using two lanes on an Illumina Hi-Seq 2000 (Illumina, San Diego, CA, United States) through Novogene (Beijing, China). Raw paired-end Illumina reads were assessed for quality using FastQC (Andrews, 2010) and were processed using Rcorrector v1.0.4 (Song and Florea, 2015) to correct random sequencing errors. Then, reads were trimmed with Trimmomatic v0.39 (Bolger et al., 2014) to remove any read containing bases with Phred scores lower than 20, low quality reads less than 50 bp long, and any adapter or other Illumina-specific sequences that were still present. The remaining reads were filtered with Kraken 2 (Wood et al., 2019) to remove Small and Large Subunit ribosomal RNA (SILVA database; Quast et al., 2013) and contaminating reads (minikraken2_v2 database). Additionally, we used custom-built databases, derived from RefSeq libraries: UniVec_Core, viral, mitochondrion, plastid, plasmid, archaea, bacteria, protozoa, human and fungi. Filtered reads were combined across all samples into a single RNA-Seq data set. We conducted a *de novo* transcriptome assembly using Trinity *v2.8.5* (Grabherr et al., 2011) to generate a single reference transcriptome assembly for *L. arvensis*. Statistical information on the assembly quality was obtained with the "TrinityStats.pl" commands in the Trinity package and Bowtie2 software (Langmead and Salzberg, 2012). Trinity produces assembled genes and isotigs (which may represent alleles, isoforms, and/or splice variants). We used the assembled genes for the DEGs in order to have a single locus per expression measure and isotigs for the SNP and phylogenetic analysis allowing a balance of certainty in the assembly quality while maximizing ortholog coverage for phylogenetic inference.

Identification of Differentially Expressed Genes (DEGs)

Since the *de novo* transcriptome assembly was done using the entire dataset as a single sample (blue- and orange-flowered plants combined), we then mapped the reads from each sample back onto the reference transcriptome. Trimmed mean of M-values (TMM, mean of log-expression ratios; Robinson and Oshlack, 2010), were calculated for each gene with RSEM software (Li and Dewey, 2011) using a Pearl script (*align_and_estimate_abundance.pl*) provided with the Trinity distribution. Then, we used EdgeR package (Robinson et al., 2009) in R v4.0.0 (R Core Team, 2020) to determine statistically significant differentially expressed genes (DEGs) between different colored samples. In order to be conservative in DEG identification, we set two thresholds for determining significant differential expression: the false discovery rate (FDR) was less than 10^{-5} and the expression difference threshold was greater than one $\log_2$ fold-change ($\log_2$FC). Wilcoxon rank-sum test for ABP and non-ABP gene comparisons were conducted in R v4.0.0 (R Core Team, 2020).

In order to focus on a broadly defined set of genes associated with the broader flavonoid biosynthetic pathway, we explored 94 structural loci (**Supplementary Table 1**). These genes start at the phenylpropanoid biosynthetic pathway and continue through the entire flavonoid biosynthetic pathway to include the ABP and three non-anthocyanin flavonoid side-branches: flavone, flavonol and isoflavonoid biosynthetic pathways (KEGG pathway database[1]). In addition, we selected 46 regulatory genes involved in flavonoid pathway regulation, including the gene families for R2R3-Myb, bHLH and WD40 loci (Li, 2014; Xu et al., 2015; Gates et al., 2017; **Supplementary Table 2**).

To identify and annotate the flavonoid biosynthetic pathway genes in our *de novo* transcriptome assembly, we used Kakapo[2]. As input, we specified a list of 140 Genbank accessions for the genes involved in flavonoid biosynthetic pathway, restricted to the Order Ericales (if the gene was not found within Ericales, we removed the Order restriction).

Phylogenetic Analysis of *F3′5′H* and *DFR*

Based on previous studies and our expression results, we carefully examined the RNA sequences of *F3′5′H* and *DFR* in relation to a set of Genbank reference sequences. In order to generate sequences of the coding region for these two enzymes, we mapped the Illumina reads of every sample to each *F3′5′H* or *DFR* isotig that arose from our Trinity *de novo* assembly. We used the "Map to Reference" function in Geneious v9.0.4 (Kearse et al., 2012), applying "highest sensitivity/slow" and "trim sequences" parameters. A subset of these isotigs, across orange- and blue-flowered samples for each gene, were used as queries in BLASTn tool from NCBI[3] to locate homologous loci from a group of reference sequences (see results section). We selected a set of Genbank references that represented the top hits and some of the best-studied model systems in flower color research for our phylogenetic analysis. For each gene, the isotigs and Genbank reference sequences were aligned using the MUSCLE plug-in from within Geneious v9.0.4. Isotigs missing large portions of the coding sequence or containing >30% ambiguities were removed from the alignment, thus excluding some putative splice variants to improve accuracy. For *DFR*, we removed the 5′ and 3′ ends (24 and 52 bp, respectively) of the coding sequence (CDS) due to alignment ambiguities before conducting phylogenetic analysis.

[2]https://github.com/karolisr/kakapo

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

We reconstructed the relationships using the RAxML 7.2.8 plug-in from within Geneious v9.0.4, searching for the maximum likelihood tree with 100 bootstrap replicates. We then interpreted the phylogenetic tree in light of normalized expression values (TMM) in R v4.0.0 (R Core Team, 2020).

SNP Frequencies for *F3′5′H* and *DFR*

In addition, we examined the frequency of non-synonymous SNPs that showed some tendency to differentiate flower color types for *F3′5′H* and *DFR-1* (*DFR-2* was only present in orange-flowered individuals so no sequences were available from blue-samples for comparison). For each sample, every isotig was determined to be fixed or variable. Although *L. arvensis* is tetraploid, we treated these as either homozygotes or heterozygotes (respectively) for our frequency calculations. We calculated the frequency of each non-synonymous SNP that showed differences between the flower color types providing BLOSUM62 scores (Henikoff and Henikoff, 1992), an empirical measure that estimates the biological probability of an amino acid substitution based on an alignment, and noting the biochemical properties of the amino acids involved. Furthermore, we extended this survey to include synonymous color-differentiating SNPs in the CDS for *F3′5′H* and *DFR-1*, including SNPs in the 5′ and 3′ UTRs for *F3′5′H*. In addition, since there were two copies of *DFR*, we also examined all non-synonymous amino acid substitutions between *DFR-1* and *DFR-2* in light of the location of variation in known functional domains responsible for substrate specificity and binding (Johnson et al., 2001; Petit et al., 2007; Smith et al., 2012). For both genes the amino acid residue numbers are based on the *L. arvensis* alignment, which translates to *Vitis vinifera* plus two amino acids (Petit et al., 2007).

Identification of Flavonoid Compounds by UHPLC-MS

Flavonoids were extracted by placing 10–15 petals of blue and orange *L. arvensis* flowers in microtubes containing 200 μl of MeOH with 1% HCl. Homogenization was performed with a BeadBeater using 3 mm × 3.2 mm steel balls for 30 s. Samples were stored at −80°C until they could be loaded on a Dionex UltiMate 3000 ultra-high pressure liquid chromatography system equipped with a diode array detector and connected to a Thermo Fleet LCQ mass spectrometer (purchased from Thermo Fisher Scientific, Waltham, MA, United States). Data were analyzed using Xcalibur software (Thermo Fisher Scientific).

Crude extracts were eluted through a Phenomenex Gemini Column (50 mm × 2 mm, 3 micron; acquired from Phenomenex, Torrance, CA, United States) with a multi-step gradient of two solvents: 0.5% formic acid in water (solvent A) and 0.5% formic acid in methanol (solvent B) at a flow rate of 0.5 mL/min. The multi-step gradient involved an initial 2 min elution at 15% B, followed by a linear gradient over 5 min to 50% B, then isocratic elution at 50% B for 3 min, then flushing the column with 100% B for 3 min. The diode array detector collected UV-Vis spectra of eluted compounds from 190–700 nm. Mass spectra of eluted compounds were ionized using electrospray ionization and the mass detector was set to positive ion detection mode with a mass range of 150–800 Da.

Flavonoid identification was based on the compound's retention time, UV-Vis spectra and whenever possible, chromatographic comparisons with authentic standards. We used flavonoid standards including quercetin, luteolin, kaempferol, isorhamnetin, malvidin, pelargonidin, cyanidin and delphinidin (purchased from Extrasynthese, Genay, France) that were previously reported for *L. arvensis* and other *Lysimachia* species (**Supplementary Table 3**). Putative identifications were also compared with published flavonoid data[4].

RESULTS

RNA Extraction and *de novo* Assembly

Bioanalyzer results confirmed we extracted high-quality RNA without substantial degradation. All RNA integrity numbers (RIN) were above the recommended RIN = 6.3 (Novogene, unpublished). The mean RIN value was 8.0 (6.7–9.0). The total number of filtered reads in the transcriptome analysis ranged from 21 million to 41 million in blue samples (mean of 26 million) and from 21 million to 26 million in orange samples (mean of 24 million). Approximately 98% of filtered reads of each sample mapped to the *de novo* assembled *L. arvensis* reference transcriptome. Isotig number per sample varied from 146 thousand to 193 thousand and the number of genes ranged from 37 thousand to 42 thousand (**Supplementary Table 4**). The GC percentage of the assembly was 38.27% and it had an N50 value of 1,635 bp. The average contig length was 973.5 bp and the total number of assembled bases equaled 389,888,932 bp. A total of 189,189 trinity genes and 400,523 trinity isotigs were obtained.

Identification of Differentially Expressed Genes (DEGs)

Initially, we identified 48,306 differentially expressed genes and 102,750 differentially expressed isotigs in the *L. arvensis* transcriptome with EdgeR. After applying the cut-off values for FDR and $\log_2$FC, we reduced the number of DEGs to 10,624 genes. There were 6,966 DEGs with higher expression in blue samples and 3,658 DEGs with higher expression in orange samples (**Supplementary Figure 2**). The average $\log_2$FC of DEGs for blue > orange (B > O) was 2.39 and for O > B was 3.16.

Restricting our analysis to the flavonoid biosynthetic pathway, our Kakapo results identified 60% (58/94) of the structural genes and 20% (10/46) of the regulatory genes in our 140 gene panel. Since some loci were found in multiple copies, a total of 153 structural gene copies and 13 regulatory gene copies were examined for differential expression (**Supplementary Tables 5**, **6**; these tables contain the number of copies for each gene identified). Gene expression analysis showed 38 structural genes and one regulatory gene with significant differential expression between flower colors (**Figure 1** and **Table 1**). Among the DEGs, 22 showed higher expression in blue-flowered plants, while 17 showed higher expression in orange-flowered plants (**Figure 1**).

[4]http://metabolomics.jp/wiki/Index:FL

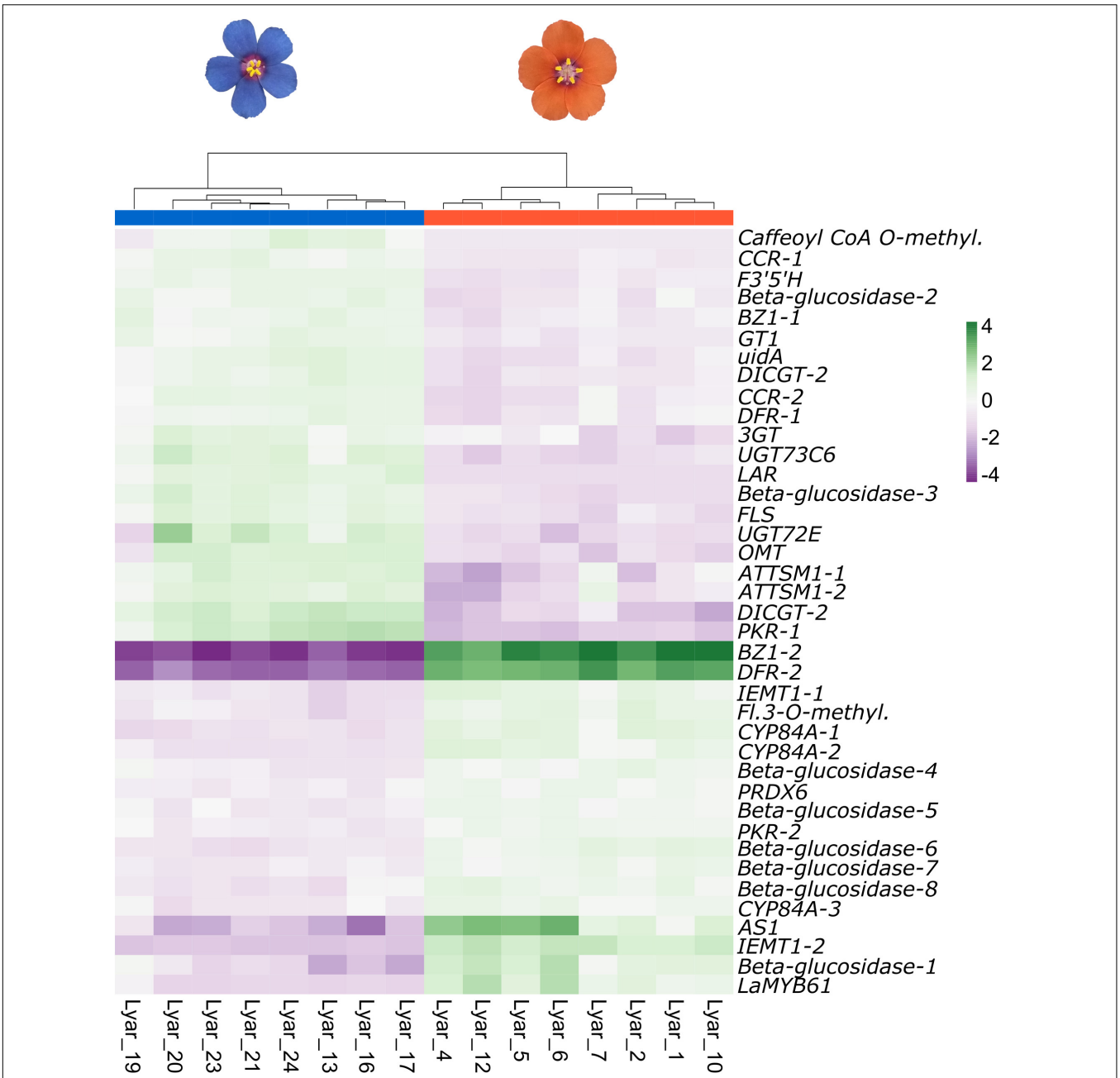


FIGURE 1 | Clustering and heatmap of differentially expressed genes (DEGs) from the flavonoid biosynthesis pathway in blue- and orange-flowered plants of *L. arvensis*. The color scale represents the scaled TMM expression values (trimmed mean of *M*-values). Green represents high expression and purple represents low expression.

Structural DEGs from the early steps of the flavonoid biosynthetic pathway have lower expression levels (TMM) compared to DEGs from the narrow ABP (Wilcoxon rank-sum test, $W = 46606$, $p = 2.2 \times 10^{-16}$; **Figure 2**). These trend is also present within flower colors (orange ABP gene expression is 4.8× higher than orange non-ABP gene expression; Wilcoxon rank-sum test, $W = 12111$, $p = 1.3 \times 10^{-12}$; blue ABP gene expression is 6× higher than blue non-ABP gene expression; Wilcoxon rank-sum test, $W = 11278$, $p = 8.5 \times 10^{-9}$). In general, structural DEGs with higher expression in blue samples tended to have ∼2× higher overall expression than those genes with higher expression in orange samples (average $TMM_{blue} = 55$ vs. average $TMM_{orange} = 29$; **Figure 2**). Focusing on the narrow ABP, we obtained eight structural DEGs with higher expression in blue *L. arvensis* (**Table 1** and **Figure 2**). On the contrary, we only found two with higher expression in orange *L. arvensis* (**Table 1** and **Figure 2**). Two candidate genes involved in the blue to orange transition had significant differential expression (*F3′5′H*

and two copies of *DFR*). *F3′5′H* and *DFR-1* show 2.5× and 2× B > O, respectively, while *DFR-2* has > 600× O > B (this locus is nearly undetectable in blue flowers). In regard to the regulatory genes, we found 13 trinity genes from four different gene families (**Supplementary Figure 3** and **Supplementary Table 6**). Only *MYB61* (*MYB4-2*) showed significant differential expression with 26× higher expression in orange- than in blue-flowered plants (**Figure 1** and **Table 1**). This MYB was initially identified as a *MYB4* from *Hibiscus syriacus* by Kakapo, but a BLASTn of this *L. arvensis* sequence showed that it is more similar to *Camellia sinensis MYB61,* and will be referred to as *LaMYB61* from here onward.

Phylogenetic Analysis of *F3′5′H* and *DFR*

Our reference-guided assembly produced complete CDS for the 67% of *F3′5′H* isotigs and 30% of *DFR-1* isotigs, both in blue- and orange-flowered samples, while the 100% of *DFR-2* isotigs were exclusively identified in orange-flowered

TABLE 1 | Flavonoid biosynthetic pathway genes with significant differential expression between blue- and orange-flowered *L. arvensis*.

Gene abbreviation	Gene name	Expression in blue (TMM)	Expression in orange (TMM)	Log fold-change	Pathway*
BZ1-2	*Anthocyanin 3-O-glucosyltransferase*	0.31	294.55	−9.81	ABP
DFR-2	*Dihydroflavonol 4 reductase*	0.15	99.80	−9.29	ABP
IEMT1-2	*(Iso)eugenol O-methyltransferase*	0.05	9.34	−7.59	Non-ABP
CYP84A-2	*Ferulate 5-hydroxylase*	0.08	2.25	−4.74	Non-ABP
LaMYB61	*Transcription factor MYB61*	0.20	5.27	−4.68	Regulatory
AS1	*Aureusidin synthase*	4.98	90.89	−4.20	Non-ABP
CYP84A-1	*Ferulate 5-hydroxylase*	0.66	4.72	−2.84	Non-ABP
Beta-glucosidase-7	*Beta-glucosidase*	0.19	1.29	−2.69	Non-ABP
Beta-glucosidase-1	*Beta-glucosidase*	15.47	83.41	−2.45	Non-ABP
Beta-glucosidase-8	*Beta-glucosidase*	0.47	2.44	−2.42	Non-ABP
PRDX6	*Peroxiredoxin 6,1-Cys peroxiredoxin*	0.28	1.29	−2.17	Non-ABP
IEMT1-1	*(Iso)eugenol O-methyltransferase*	7.42	24.85	−1.77	Non-ABP
CYP84A-3	*Ferulate 5-hydroxylase*	0.87	2.84	−1.72	Non-ABP
Beta-glucosidase-5	*Beta-glucosidase*	0.66	2.01	−1.63	Non-ABP
Fl. 3-O-methyl.	*Flavonol 3-O-methyltransferase*	7.26	21.53	−1.59	Non-ABP
Beta-glucosidase-6	*Beta-glucosidase*	6.94	20.24	−1.56	Non-ABP
Beta-glucosidase-4	*Beta-glucosidase*	1.75	4.14	−1.26	Non-ABP
PKR-2	*Polyketide reductase*	1.66	3.80	−1.20	Non-ABP
DFR-1	*Dihydroflavonol 4 reductase*	406.80	193.69	1.06	ABP
CCR-1	*Cinnamoyl CoA reductase*	50.45	22.93	1.12	Non-ABP
BZ1-1	*Anthocyanin 3-O-glucosyltransferase*	58.20	25.46	1.18	ABP
F3′5′H	*Flavonoid 3′5′-hydroxylase*	655.06	267.30	1.28	ABP
DICGT-1	*Chalcononaringenin 2′-O-glucosyltransferase*	39.46	14.95	1.39	Non-ABP
GT1	*Anthocyanin 5,3-O-glucosyltransferase*	6.88	2.47	1.47	ABP
3GT	*Anthocyanin 3′-O-beta-glucosyltransferase*	20.51	7.14	1.50	ABP
CCR-2	*Cinnamoyl CoA reductase*	7.64	2.40	1.66	Non-ABP
uidA	*Beta glucuronidase*	15.59	4.82	1.68	Non-ABP
FLS	*Flavonol synthase*	503.38	144.86	1.77	Non-ABP
ATTSM1-2	*Caffeoyl CoA 3-O-methyltransferase*	28.12	7.53	1.89	Non-ABP
Beta-glucosidase-2	*Beta-glucosidase*	3.36	0.85	1.96	Non-ABP
ATTSM1-1	*Caffeoyl CoA 3-O-methyltransferase*	18.57	3.84	2.26	Non-ABP
Beta-glucosidase-3	*Beta-glucosidase*	12.76	2.62	2.26	Non-ABP
OMT	*O-methyltransferase*	145.81	27.40	2.39	ABP
UGT72E	*Coniferyl alcohol glucosyltransferase*	17.68	2.26	2.94	Non-ABP
DICGT-2	*Chalcononaringenin 2′-O-glucosyltransferase*	46.75	5.20	3.16	Non-ABP
UGT73C6	*Flavonol 3-O-L-rhamnoside-7-O-glucosyltransferase*	5.89	0.51	3.50	Non-ABP
PKR-1	*Polyketide reductase*	12.16	0.40	4.88	Non-ABP
LAR	*Leucoanthocyanidin reductase*	3.28	0.09	5.25	Non-ABP
Caffeoyl CoA O-methyl.	*Caffeoyl CoA-O-methyltransferase*	1.27	0.00	7.65	Non-ABP

Expression is calculated by the average TMM values per gene per each color.
Negative Log fold-change values indicate a higher expression in orange samples, while positive values indicate higher expression in blue samples.
**ABP: genes that belong to the narrow ABP; Non-ABP: genes that belong to the broader flavonoid biosynthetic pathway, excluding ABP genes; Regulatory genes indicated.*

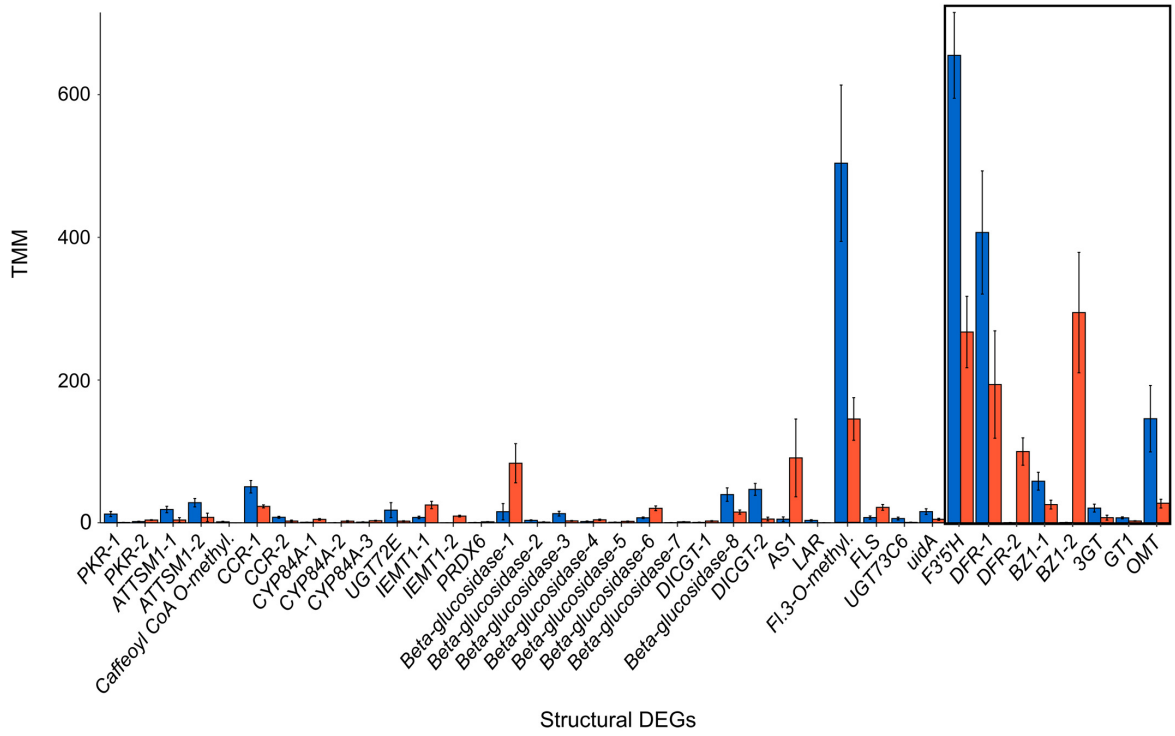


FIGURE 2 | Expression of structural DEGs of the flavonoid biosynthesis pathway with significant differential expression between blue and orange *L. arvensis*. The colored bars represent the average expression level in TMM (trimmed mean of M-values) units and error bars show standard deviation. The black box indicates DEGs from the narrow ABP.

samples. The phylogenetic analysis of *F3′5′H* produced a tree with monophyletic clade of *L. arvensis* samples sister to *Cyclamen graecum* (Accession QG891056) and closely related to several *Camellia sinensis* samples (**Figure 3**). Isotigs cluster in monophyletic or nearly monophyletic clades for each flower color (e.g., orange-i2 and orange-i10 form a clade; a large clade with nearly all blue samples). There are no signs of gene duplication within *L. arvensis F3′5′H*. Sample L19 (blue) may be a hybrid between blue and orange (even though these are inbred lines) since for some isotigs, L19 clusters with orange samples (i1, i4, i3) and for other isotigs, it arises from the base of a clade of blue-flowered samples (i2, i10).

The phylogenetic analyses of the two forms of *DFR* indicate that there are two distinct lineages of *DFR* (*DFR-1* and *DFR-2*) and that *L. arvensis* samples of each are both reciprocally monophyletic yet the two *DFR*s are deeply diverged within angiosperms (i.e., *DFR-1* and *DFR-2* diverged long before the origin of *Lysimachia*) (**Figure 4** and **Supplementary Figure 4**). The clade of orange-flowered *DFR-2* samples is very strongly supported (100% bootstrap support). It is sister to several Genbank reference samples from the Asterids (members of the Asteraceae, Solanaceae, Convolvulaceae, Plantaginaceae). Alternatively, *DFR-1* forms a very strongly supported clade (100% bootstrap) that is closely related to two samples from the Ericales (*Cyclamen graecum* and *Camellia spp.*). There is very strong support for a clade containing *DFR-1 Camellia* and *Cyclamen* to the exclusion of *DFR-2* and the Asterid samples (100% bootstrap). There are 74 non-synonymous amino acid changes separating *DFR-1* and *DFR-2*. Five of these are variable in *DFR-1*, but fixed in *DFR-2* and one is variable in *DFR-2*, but fixed in *DFR-1*. This includes five amino acid replacements in known functional domains (see "SNP frequencies" subsection below). Since there were no blue *DFR-2* sequences created during the reference-guided assembly, all further analyses comparing orange- and blue-flowered samples focus on *DFR-1*.

For the *DFR-1* clade, there are two patterns. The first pattern clusters isotigs into monophyletic clades with other isotigs of the same color (e.g., orange-i10, i2 and i8 form a strongly supported clade and blue-i10 and blue-i2 form a moderately supported clade; **Figure 4**). The second pattern consists of blue samples of one isotig which are sister to the orange samples from the same isotig (e.g., i7 and i11). Similar to the results for *F3′5′H*, the blue sample L19 falls within orange clades for all isotigs, sometimes with very strong bootstrap support, consistent with it being a hybrid.

SNP Frequencies for *F3′5′H* and *DFR*

We investigated SNP frequencies that correlate with flower color in two candidates within the narrowly defined ABP genes. For *F3′5′H*, there were four non-synonymous SNPs in the CDS (**Figure 5A**). Two of these were fixed for all orange samples in all isotigs and variable in all blue samples for all isotigs (bp472 and bp1426; **Table 2**). Both had BLOSUM62 scores of +1 (meaning relatively common amino acid replacements), yet

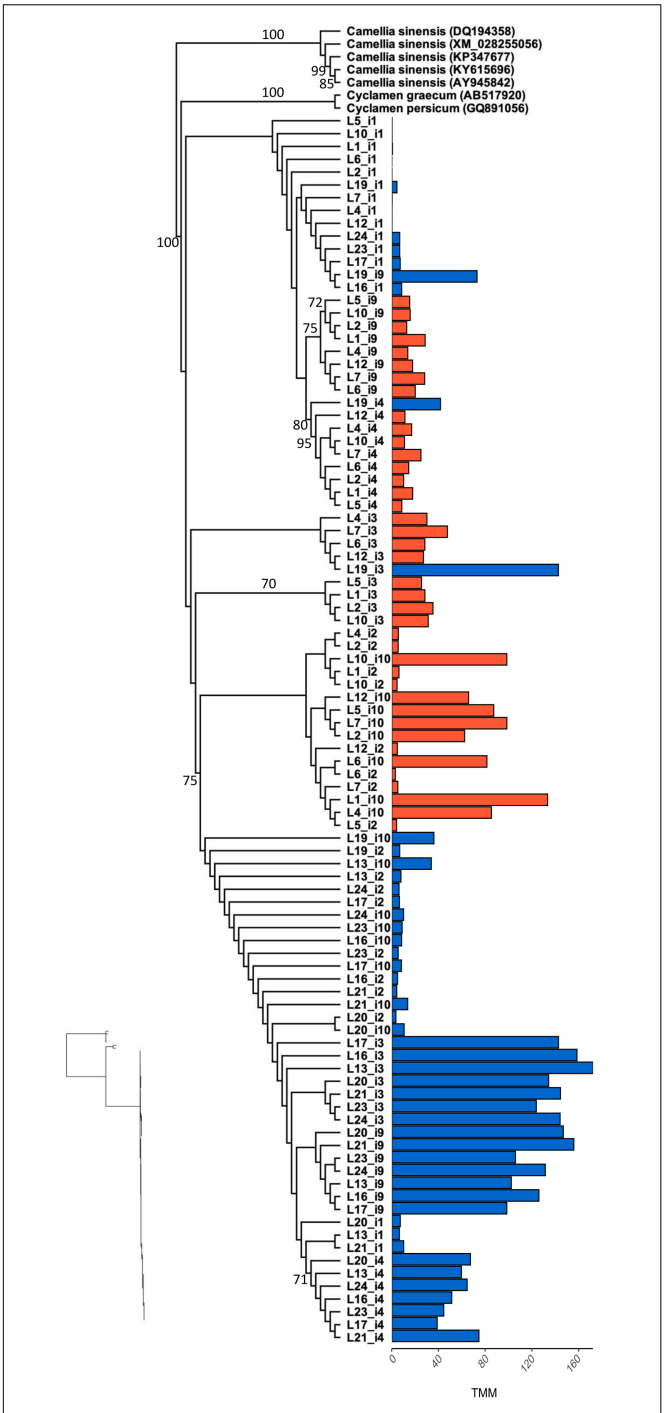


FIGURE 3 | Maximum likelihood phylogenetic analysis of *F3′5′H* coding sequence for all *L. arvensis* isoforms (L1–12 = orange; L13–24 = blue). We included the top BLASTn hits for reference (scientific name followed by followed by Genbank Accession numbers). Bootstrap values are provided along the branch or at the node for values above 70%. The bar plot shows the expression value (TMM) per isotig for blue and orange flower colors. The isotigs with markedly shorter coding sequences than the Genbank references are *F3′5′H-i1* and *F3′5′H-i4*. The maximum likelihood phylogram without tip labels (yet same order) is included to allow for relative branchlength comparisons.

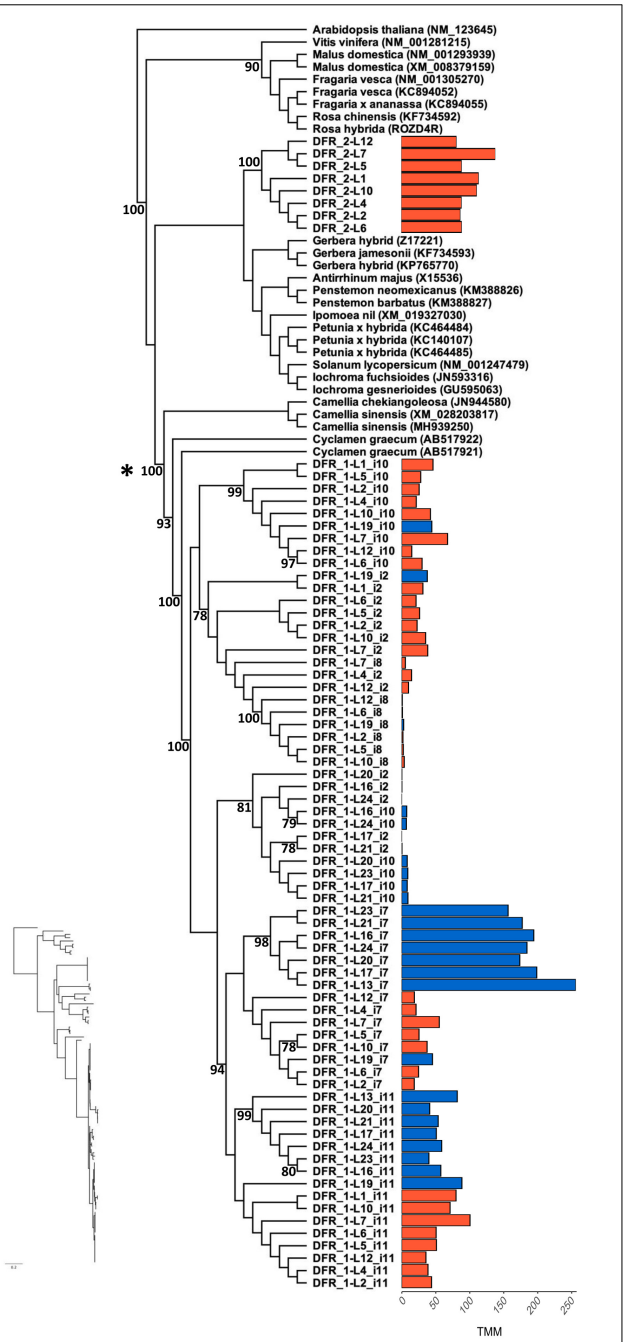


FIGURE 4 | Maximum likelihood phylogenetic analysis of *DFR-1* and *DFR-2* coding sequence for all *L. arvensis* isoforms (L1–12 = orange; L13–24 = blue). We included the top BLASTn hits for reference (scientific name followed by followed by Genbank Accession numbers). Bootstrap values provided along the branch or at the node for values above 70%. We removed he 5′ and 3′ ends (24 and 52 bp, respectively) of the coding sequence because of alignments ambiguities. The bar plot shows the expression value (TMM) per isotig for blue and orange flower colors. The isotigs with markedly shorter coding sequences than the Genbank references are *DFR-1-i7*, *DFR-1-i8*, and *DFR-1-i11*. *Indicates the location of the putative gene duplication event. The maximum likelihood phylogram without tip labels (yet same order) is included to allow for relative branchlength comparisons.

each of them involved significant changes in the biochemistry of the amino acids involved (e.g., the SNP at bp472 changes from a positively charged H to a polar uncharged N and the SNP at bp1426 changes from a positively charged K to a negatively charged E). There were 18 additional synonymous SNPs in the *F3′5′H* CDS most of which were variable in blue samples, but not in orange samples (**Supplementary Table 7**). Over all 22 *F3′5′H* SNPs (synonymous and non-synonymous), the frequency differences between blue- and orange-flowered samples (across isotigs) ranged from 0.09 to 0.83 (mean 0.53). There were an additional five SNPs in the 5′-UTR and six SNPs in the 3′-UTR. The average difference between blue- and orange-flowered SNP frequencies in the UTRs was 0.57 (0.09–0.79). None of the non-synonymous SNPs for *F3′5′H* were located in any of the known functional domains (**Figure 5A**).

Since there are well-characterized functional domains in *DFR* that provide substrate specificity conferring blue-to-red shifts in other species (Johnson et al., 2001; Petit et al., 2007; Des Marais and Rausher, 2008; Smith et al., 2012), we compared *DFR-1* and *DFR-2* for fixed amino acid differences at these sites. For the 26 residue substrate specificity region identified by Johnson et al. (2001) (amino acids 133 to 158 in our alignment), the *L. arvensis DFR-2* has fixed differences in 13 of them compared to *DFR-1* (**Figure 5B**). For the additional 12 substrate binding sites identified by Petit et al. (2007), we found only one site (amino acid 210) where *DFR-2* was fixed with a serine (S) compared to a threonine (T) in *DFR-1* (BLOSUM62 score = +1; both polar uncharged amino acids). Two more color-differentiating sites identified by Smith et al. (2012) were fixed in both *DFR*s (**Figure 5B**). In addition to these five amino acid substitutions in known functional domains, we identified 63 more amino acid changes outside known functional domains that were fixed between *DFR-1* and *DFR-2* and another six that were variable in one copy of *DFR*, yet fixed in the other.

Since *DFR-2* is only expressed at considerable levels in orange flowers, we focus our color-differentiating SNP analysis on *DFR-1* where we have sequences of both blue- and orange-flowered individuals. Over all 54 *DFR-1* SNPs (synonymous and non-synonymous), the frequency differences between blue- and orange-flowered samples (across isotigs) ranged from 0.12 to 1 (mean 0.46). There were ten non-synonymous SNPs with considerable differences in frequency between flower colors (**Table 3**). The non-synonymous SNPs located in the binding specificity region (bp434 and bp474; **Figure 5B**) showed clear patterns of color frequency differences between the two flower color types (bp434 = 0.50 SNP frequency difference; bp474 = 0.52 SNP frequency difference; **Supplementary Table 8**). Both have a BLOSUM62 score of +1 and are amino acid substitutions within the same general biochemical category (bp434 = both polar uncharged amino acids; bp474 = both hydrophobic amino acids). Three non-synonymous SNPs in the 3′ half of the *DFR-1* coding sequence that (only present in i2 and i10) were completely differentiated between blue- and orange-flowered samples (bp859, bp863, and bp959). Their BLOSUM62 scores range from 0 to +2 and two of them represent biochemically distinct amino acid replacements (bp859 and bp913 = negatively charged to polar uncharged amino acids; **Table 3**). There were 44 additional synonymous SNPs in the *DFR-1* CDS (**Supplementary Table 8**).

Identification of Flavonoid Compounds by UHPLC-MS

Ultra-High Performance Liquid Chromatography analysis confirmed that malvidin-3-rhamnoside is the main flavonoid that accumulates in the blue petals of *L. arvensis* (relative content = 98.4%; **Table 4** and **Figure 6**). In addition, blue petals also contain traces of the aglycone malvidin and delphinidin derivatives. In contrast, orange petals contain four derivatives of pelargonidin with different glycosylation patterns: pelargonidin-3-glucoside (60.1% relative content), pelargonidin-3-glucoside-glucuronide (26.5%), pelargonidin-3-rhamnoside (11.6%), and pelargonidin-3-diglucoside (trace amounts; **Table 4** and **Figure 6**). We also detected traces of the flavone luteolin-7-glucoside in the blue samples, and small amounts of two flavonols present in some orange samples – syringetin (trace) and kaempferol-3-glucoside (1.7%). The putative locations of these compounds in the flavonoid biosynthetic pathway are shown in **Figure 7**.

DISCUSSION

We found 38 genes with significant differential expression between flower colors of *L. arvensis* across 94 genes in the flavonoid biosynthetic pathway. Our two sampled populations are geographically separated and may represent incipient species based on recent phylogenetic results (Jiménez-López, 2019; Jiménez-López et al., in review), which may have introduced some differences between the populations not correlated with color. Nevertheless, we still detected patterns correlated with color that are consistent with known functions of the ABP loci and previous studies of comparable flower color transitions. First, we focus on the ABP genes including discussion about the expression differences, SNP detection and biochemical changes associated with two enzymes at a critical branchpoint in the ABP in order to shed light on the molecular underpinnings of the blue to orange shift in *L. arvensis*. Then, we expand the conversation to include additional genes in the flavonoid biosynthetic pathway not previously associated with such a color shift and discuss potential pleiotropic consequences not directly related to pollinator attraction. Finally, we mount evidence for a directional transition from blue to orange flowers in *L. arvensis*.

Within the narrow ABP, there were two DEGs in *L. arvensis* concentrated in the latter half of the pathway that are likely directly involved in the blue to orange shift in flower color (*F3′5′H* and *DFR*). These genes had the highest overall expression of all the narrowly defined ABP genes. This flower color transition could be caused by either of these or some combination of the two since these genes are responsible for the synthesis of two different types of anthocyanins: delphinidin-derivatives (in the case of *L. arvensis* this is malvidin that produces the blue color) and pelargonidin-derivatives (orange; **Figure 7**; Smith and Rausher, 2011; Smith et al., 2012; Wheeler and Smith, 2019). These biochemical results are consistent with previous

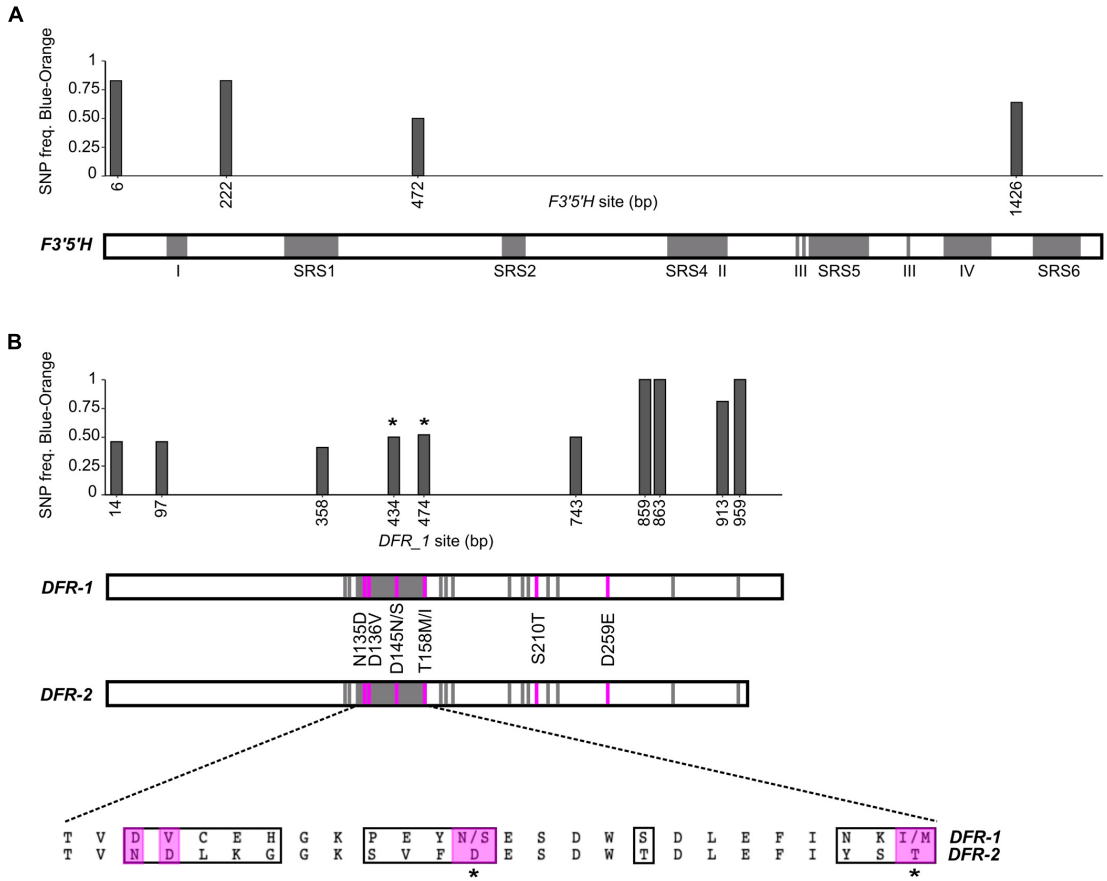


FIGURE 5 | Non-synonymous SNP analysis of *F3′5′H* and DFR for *L. arvensis*. SNP frequency graphs are aligned with the gene diagram indicating known functional domains (gray). For *F3′5′H* **(A)** there are five binding regions (SRS; Seitz et al., 2007) and other known functional domains (I-IV; Guo et al., 2019). The DFR analysis **(B)** shows non-synonymous SNP frequencies in DFR-1 in the graph. Gene diagrams show the substrate specificity region (amino acid sites 133–158; Johnson et al., 2001) and other previously identified substrate binding sites (Petit et al., 2007; Smith et al., 2012) in gray. Amino acid changes between blue and orange DFR-1 located in a substrate binding region are marked with *. Amino acid changes between DFR-1 and DFR-2 identified in other works to be relevant to the substrate specificity differentiation between colors (Petit et al., 2007; Smith et al., 2012) are indicated in the diagram (pink) and their location and single letter abbreviation are shown between the DFR diagrams. An alignment of DFR-1 and DFR-2 consensus sequences of the 26 amino acids from the substrate specificity region is provided, highlighting all the amino acid changes in a black box (sites 133-158; relevant amino acid changes shown in the gene diagram are highlighted in pink).

findings in *L. arvensis* (Lawrence et al., 1939; Harborne, 1968; Ishikura, 1981; Kawashty et al., 1998). Several additional ABP genes with differential expression in blue- vs. orange-flowered plants were consistent with the post-translational modifications of these principle pigments. Here, we discuss three of these genes (**Figure 2**). *BZ1-2* showed high levels of expression in orange petals (TMM > 290) compared to blue (expression 950× O > B). This enzyme introduces a glucose group to the 3-position of the anthocyanin precursor's C-ring to stabilize the color (Koes et al., 2005). The high expression of *BZ1-2* in orange-flowered *L. arvensis* plants correlates with the biochemical results showing that orange petals are composed of pelargonidin 3-glucoside derivatives whereas blue petals are malvidin glycosylated with rhamnoside. Similarly, enzymes *OMT* and *3GT* showed higher levels of expression in blue than orange samples (5.3× and 2.9×, respectively). *OMT* methylates delphinidin to form malvidin, and *3GT* introduces the rhamnoside (Saito et al., 2013) both of which are unique to blue flowered samples, likely involved in stabilization and protection of the pigment, but unlikely solely responsible for the color change.

Role of *F3′5′H* Expression and Amino Acid Substitutions

F3′5′H catalyzes the hydroxylation of dihydrokaempferol to produce a delphinidin precursor (Halbwirth, 2010). Our expression results indicate a 2.5× decrease in expression in orange petals consistent with several previous studies investigating the blue- to orange-flowered shift among a diversity of eudicots (Rausher, 2008; Wessinger and Rausher, 2014; Larter et al., 2018). For example, the loss of function of *F3′5′H* in *Iochroma* spp. (Smith and Rausher, 2011), *Antirrhinum* spp. (Ishiguro et al., 2012) and *Penstemon barbatus* (Wessinger and Rausher, 2013) or the decreased expression of *F3′5′H* in *Phlox drummondii* (Hopkins and Rausher, 2011), confers blue to red color shifts. Rausher (2008) suggests that unidirectional changes

at this key step in the ABP play an important role in the repeated transitions from blue to red/orange flowers observed in many angiosperm lineages. In *L. arvensis*, *F3′5′H* activity is not completely lost since *F3′5′H* isotigs were detected (at low levels) in orange petals and we detected traces of a flavonol requiring *F3′5′H* activity (syringetin) in almost 30% of the orange petal samples. In addition to the decreased expression of *F3′5′H*, there are four flower color differentiating non-synonymous SNPs that may contribute to the blue to orange shift, but would require future enzyme activity assays to be determined.

Role of *DFR* in the Blue to Orange Transition

The transition from delphinidin-like precursors (malvidin in this case) to pelargonidin precursors caused by changes in *F3′5′H*, creates a new substrate which requires subsequent modifications in downstream enzymes like *DFR*. Several lines of evidence suggest that *DFR* in *L. arvensis,* which catalyzes the reduction of dihydroflavonols to leucoanthocyanins, may also be involved in the evolutionary transition from blue to orange flowers. Our transcriptome assembly identified two distinct copies of *DFR*. One copy, *DFR-2*, is expressed primarily in orange flowered individuals (nearly undetectable in blue samples). This copy presents an N residue in the third position of the substrate specificity region (residue 135 in *L. arvensis*), which is known to confer substrate affinity to dihydrokaempferol, the pelargonidin-like precursor, in a wide group of angiosperms (Johnson et al., 2001; Shimada et al., 2005). Alternatively, *DFR-1* is primarily expressed in blue-flowered individuals and exhibits a D residue at this site, which confers less or no substrate affinity for dihydrokaempferol in other species (Johnson et al.,

TABLE 2 | Comparison of amino acid (AA) variation in the *F3′5′H* non-synonymous SNP sites of the coding sequences between blue and orange *L. arvensis* isotigs.

	Isotig	Site 6	Site 222	Site 472	Site 1426
Blue*	i1	E	K	N/H	K/E
	i2	E	K	N/H	K/E
	i3	E	K	N/H	K/E
	i4	E	K	N/H	K/E
	i9	E	K	N/H	K/E
	i10	E	K	N/H	K/E
Orange	i1	E	K	N	E
	i2	E	K	N	E
	i3	E	K	N	E
	i4	D	N	N	E
	i9	E	K	N	E
	i10	E	K	N	E
BLOSUM62 score		+2	0	+1	+1
Biochemistry		Both negatively charged	Positive (K) to polar uncharged (N)	Positive (H) to polar uncharged (N)	Positive (K) to negative (E)

Site numbers refer to base pairs (bp).
**Not including blue sample L19, which is closely related to orange samples.*

TABLE 3 | Comparison of amino acid (AA) variation in the *DFR_1* non-synonymous SNP sites of the coding sequences between blue and orange *L. arvensis* isotigs.

	Isotig	Site 14	Site 97	Site 358	Site 434	Site 474	Site 743	Site 859	Site 863	Site 913	Site 959
Blue*	i10	I/T	T	K/G	N/S	I/M	E	E	S	E	N
	i11	T	T	E	N	I	–	–	–	–	–
	i2	I	T	K/G	N/S	I/M	E	E	S	E	N
	i7	I	T	T	N	M	–	–	–	–	–
Orange	i10	T	T	K	S	I	A	Q	N	Q	T
	i11	T	T	K	N	I	–	–	–	–	–
	i2	I	S/T	K	S	I	E	Q	N	Q/E	T
	i7	I	S/T	T/I	N	I	–	–	–	–	–
	i8	I	S/T	K	S	I	–	–	–	–	–
BLOSUM62 score		−1	+1	NA	+1	+1	−1	+2	+1	+2	0
Biochemistry		Hydrophobic (I) to polar uncharged (T)	Both polar uncharged	NA	Both polar uncharged	Both hydrophoabic	Hydrophobic (A) to negative charged (E)	Negatively charged (E) to polar uncharged (Q)	Both polar uncharged	Negatively charged (E) to polar uncharged (Q)	Both polar uncharged

Site numbers refer to base pairs (bp).
**Not including blue sample L19, which is closely related to orange samples.*

2001; Shimada et al., 2005). We propose that *DFR-1* in *L. arvensis* is likely more efficient in processing dihydromyricetin, the delphinidin-like precursors to malvidin. Like *F3′5′H*, a combination of expression and changes in the amino acid composition of *DFR* likely contributes to the completion of the blue to orange color transition. In *Petunia*, alteration of the amino acids in the substrate specificity domain of *DFR* confers a flower color shift by increasing catalytic efficiency in metabolizing dihydrokaempferol, a pelargonidin precursor (Johnson et al., 2001). In addition, three more amino acid changes between *DFR-1* and *DFR-2* have been suggested to be involved in substrate specificity differentiation in other species. In *Iochroma* and *Vitis* (Petit et al., 2007; Smith et al., 2012), the number of sites responsible for substrate specificity is larger and more physically spread out along the *DFR* coding region than the 26 amino acid stretch suggested by Johnson et al. (2001). All these amino acid changes between the two copies of *DFR* present in *L. arvensis* are correlated with blue to orange flower color transition, yet causality would require further genetic manipulation.

Within the *DFR-1* coding sequence of *L. arvensis*, there are many color differentiating SNPs. There are two amino acid changes nearly fixed between colors that also differ from *DFR-2*, and are located in the 26 amino acid substrate specificity region, suggesting interesting residue positions for future studies of differential substrate binding affinity. Overall, we suggest that *DFR-1* may have had an original affinity for dihydromyricetin, which is maintained in blue flowers, while in orange-flowered individuals, a trans-regulatory change to *DFR-2* expression (e.g., via *LaMYB61* O > B) may confer dihydrokaempferol specificity to accommodate the new substrate being produced by the loss of *F3′5′H* activity. If *DFR-2* outcompetes *F3′* for dihydrokaempferol as described in other angiosperms producing pelargonidin (Des Marais and Rausher, 2008), then the flux would be directed to the pelargonidin pathway (even if *F3′* enzymes are present as we have relatively equal expression in blue and orange-flowered samples for *F3′*). In order to produce orange flowers, there must be no (or very low) expression of *DFR-2* in blue *L. arvensis* to allow *F3′5′H* to win the competition for dihydrokaempferol redirecting the flux down the delphinidin branch of the ABP.

The maintenance and detectable expression of both *DFR-1* and *DFR-2* in *L. arvensis* is somewhat enigmatic. A similar situation exists in the Convolvulaceae with three *DFR* copies of relatively recent origin in that family (Des Marais and Rausher, 2008). However, the duplication of *DFR-1* and *DFR-2* we report is likely independent of the gene duplications reported by Des Marais and Rausher (2008), because it appears to be much older, perhaps predating the split of Asterales and Ericales (see annotation in **Figure 4**). Next, we plan to investigate the petal transcriptomes of the closely related *L. monelli*, which exhibits phenotypically similar petal color polymorphism. We expect to find two copies of *DFR* in that case, as well, and will compare its color-specific expression differences to those we report for *L. arvensis* herein.

Broader Insights From the Flavonoid Biosynthetic Pathway and the Roles of Non-pollinator Agents of Selection

We hypothesized that there would be some flower-color specific differences in the transcriptome for genes outside the narrow ABP that might open the door for agents of selection not associated with pollinator attraction (Strauss and Whittall, 2006; Rausher, 2008; Ortiz-Barrientos, 2013). In this regard, we found 30 DEGs in the broader flavonoid pathway, outside the narrow ABP (15 DEGs with B > O with an average fold-change of 9.1 and 15 DEGs with O > B with an average fold-change of 18.8). Among these non-ABP DEGs, we looked for loci with potential functionality that may correlate with the ecological differences of the two color types (Arista et al., 2013). For example, *Caffeoyl CoA O-methyl* (only present in blue samples with TMM = 1.27) and *Caffeoyl 3-O-methyl* (*ATTSM1-1* and *ATTSM1-2*, with B > O

TABLE 4 | Putative flavonoid identification in blue and orange petals of *L. arvensis* from the UHPLC–MS biochemical analysis (MS analysis was acquired in positive mode).

Petal color	Flavonoid type	Putative flavonoid identification	N samples*	t_R (min)	λ_{max} (nm)	Mass (m/z)	Relative content (%)**
Blue	Anthocyanin	Delphinidin 3-glucoside-rhamnoside	1/14	2.3	233, 302, 522	609.90	trace
(14 plants)	Anthocyanin	Delphinidin 3-rhamnoside-rhamnoside	1/14	3.3	235, 310, 522	592.45	trace
	Anthocyanin	Malvidin 3-rhamnoside	14/14	5.8	227, 280, 527	476.71	98.4
	Anthocyanin	Malvidin	1/14	7.0	231, 268, 350, 533	331.28	1.6
	Flavone	Luteolin 7-glucoside	2/14	7.9	230, 290, 340, 377	448.48	trace
Orange	Anthocyanin	Pelargonidin 3-glucoside-glucuronide	4/11	2.2	279, 427, 502	606.66	26.5
(11 plants)	Anthocyanin	Pelargonidin 3-diglucoside	2/11	2.8	239, 280, 428, 500	594.72	trace
	Anthocyanin	Pelargonidin 3-glucoside	11/11	3.0	283, 427, 502	433.09	60.1
	Anthocyanin	Pelargonidin 3-rhamnoside	6/11	5.2	232, 268, 483, 506	416.72	11.6
	Flavonol	Syringetin	3/11	6.9	232, 264, 294, 346	346.64	trace
	Flavonol	Kaempferol 3-glucoside	5/11	7.9	232, 264, 294, 346	448.75	1.7

Petals do not include the darker basal area (i.e., bullseye).
Identification was based on retention time (t_R), maximum absorbance wavelength (λmax) observed in the UV-Vis spectra of the compound that eluted at the t_R indicated, and molecular mass comparisons with standards and values of previously reported flavonoids for Lysimachia spp. (see ***Supplementary Table 2*** *and the database http://metabolomics.jp).*
**The number of samples where each flavonoid was observed.*
***Relative content of each putative compound in each flower color, calculated from the areas of the UV-Vis peaks.*

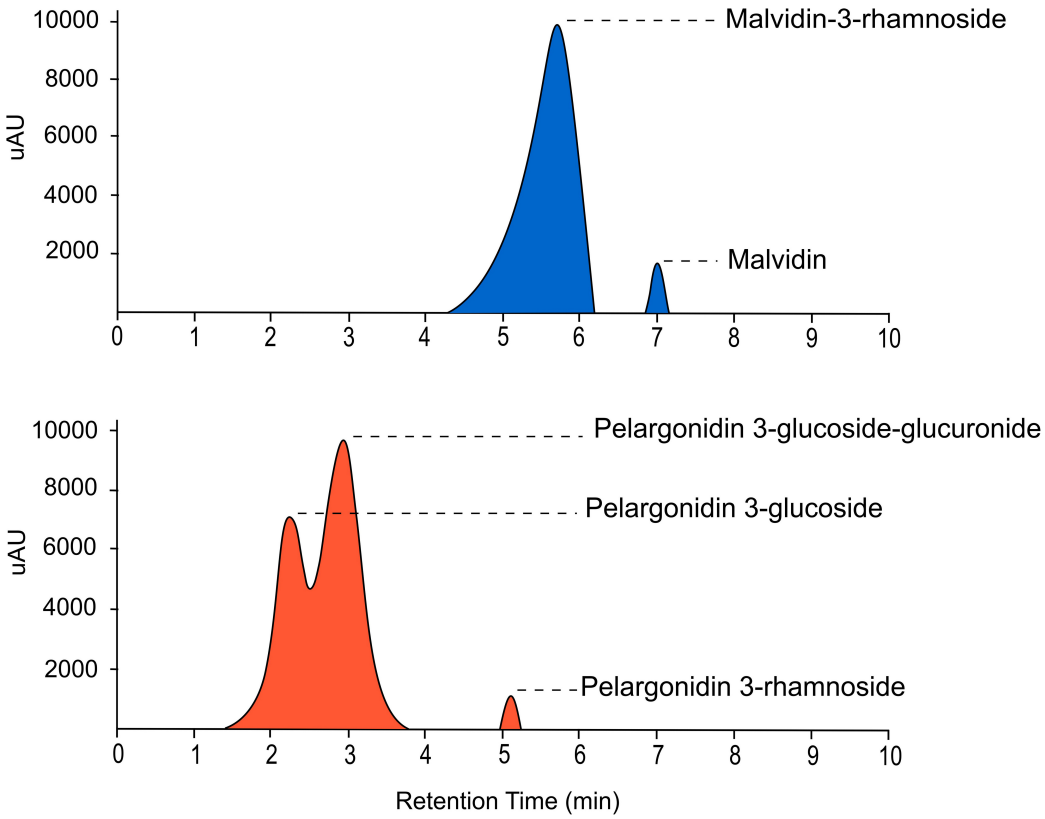


FIGURE 6 | UHPLC-MS chromatograms of the main anthocyanins present in blue (above) and orange (below) petals of *L. arvensis* detected at 520 nm.

and an average fold-change of 4.8 and 3.7, respectively) both promote anthocyanin methylation following drought stress in *Vitis vinifera* (Giordano et al., 2016) and have been implicated in lignin biosynthesis, an important barrier against pathogens (Liu et al., 2018). Additionally, we found differential expression for three *CYP84A* genes with O > B fold-changes ranging from 3.3 to 28.1. These loci are involved in sinapate ester biosynthesis, a key flavonoid compound providing UV protection (Ruegger et al., 1999). Finally, in defense against herbivores and pathogens, we found differential expression of two *IEMT* (with O > B average fold-change of 3.3 and 186.8) involved in the synthesis of isomethyleugenol and methyleugenol for deterring herbivores (and/or attracting pollinators; Barkman, 2003) and two *PKR* (also known as *CHR;* one with B > O fold-change of 30.4, and the other with O > B fold-change of 2.3) which produces phytoalexins (isoflavonoids, coumestans, pterocarpans, and isoflavans) in response to herbivore or pathogen attack in legumes (Bomati et al., 2005).

The most dramatic expression differences we found between blue and orange *L. arvensis* petal transcriptomes are those in the ABP genes. The average fold-change of all significantly differentially expressed ABP genes is 204×, but only 14× for non-ABP flavonoid pathway genes. Furthermore, most ABP overall expression levels are much higher than all other flavonoid pathway genes combined. The relative importance of the ABP vs. non-ABP genes are consistent when correcting for color-type (orange ABP gene expression is 4.8× higher than orange non-ABP gene expression; Wilcoxon rank-sum test, $p = 1.3 \times 10^{-12}$; blue ABP gene expression is 6× higher than blue non-ABP gene expression; Wilcoxon rank-sum test, $p = 8.5 \times 10^{-9}$). A notable exception to the low expression of non-ABP flavonoid pathway genes is flavonol synthase (FLS). This side-branch in the ABP catalyzes the synthesis of flavonols from dihydroflavonols. In *L. arvensis* petals, *FLS* has very high expression in blue samples (TMM > 500) and has 3.5× higher expression in blue- vs. orange-flowered samples (**Figure 2** and **Table 1**). In *Arabidopsis, FLS* has the highest expression in petals, and it can compete with *DFR* for flux down the anthocyanin portion of the ABP (Nguyen et al., 2016; McCarthy et al., 2017). It is often involved in plants' abiotic stress response (Lee et al., 2016) neutralizing damaging reactive oxygen species (Li B. et al., 2019). In contrast, activation or inactivation of *FLS* is involved in pollinator shifts in *Petunia* species (Esfeld et al., 2018). Nevertheless, our UHPLC results lack any measurable levels of flavonols (over 98% of detected flavonoids were anthocyanins in both color types; **Table 4**). This suggests that either (a) *DFR* is such a good substrate competitor that, even with high expression of *FLS*, the flux continues toward anthocyanin production, or (b) the main activity of *FLS* is not for flavonol production, but may be involved in some other undescribed pathway.

The transcriptome results for the broader flavonoid biosynthetic pathway genes are largely confirmed by our

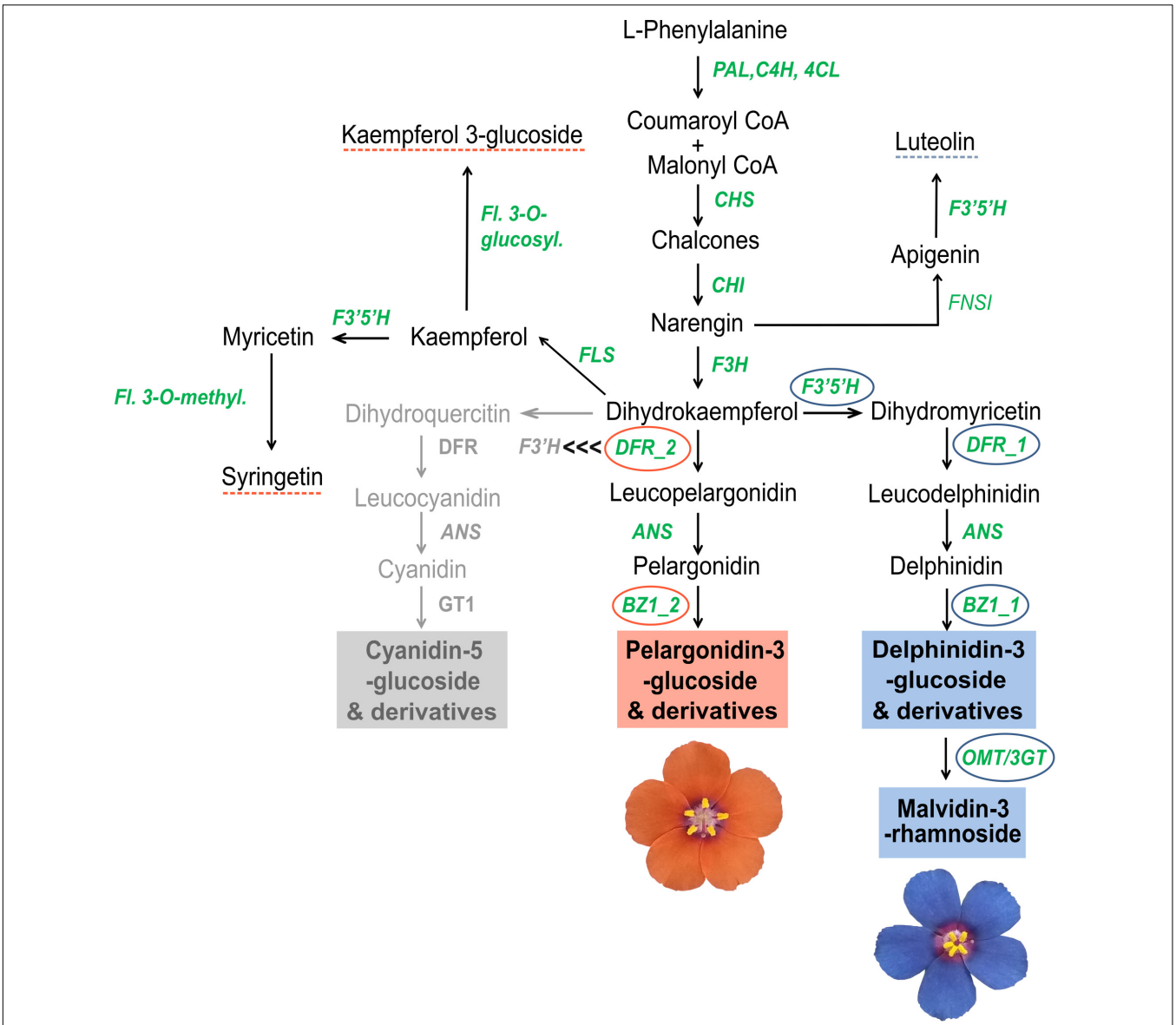


FIGURE 7 | Tentative scheme of the anthocyanin biosynthetic pathway for *L. arvensis* petals. Structural genes involved in the broader flavonoid biosynthetic pathway are shown in green: *Phenylalanine ammonia-lyase (PAL)*, *Cinnamic 4-hydroxylase* (*C4H*), *4-coumarate-CoA ligase* (*4CL*), *Chalcone synthase* (*CHS*), *Chalcone isomerase* (*CHI*), *Flavanone 3-hydroxylase* (*F3H*), *Flavonoid 3′,5′-hydroxylase* (*F3′5′H*), *Dihydroflavonol 4-reductase* (*DFR*), *Anthocyanin synthase* (*ANS*), *Anthocyanidin-3-o-glucosyltransferase* (*BZ1*), *Anthocyanin-3′-o-beta-glucosyltransferase* (*3GT*), *O-methyltransferase* (*OMT*), *Flavonol synthase* (*FLS*), *Flavonol-3-o-glucosyltransferase* (*Fl. 3-o-glucosyl.*), *Flavone synthase I* (*FNSI*), and *Flavonol 3-O-methyltransferase* (*Fl. 3-O-methyl.*). Narrow ABP genes differentially expressed in orange and blue *L. arvensis* are marked with orange and blue circles, respectively. Blue and orange boxes mark the anthocyanin compounds found by UHPLC-MS in each flower color. Blue and orange dashed lines mark the non-anthocyanin compounds found by UHPLC-MS in each flower color. The gray portion indicates the location of the inactivated branch leading to cyanidin-derivatives that would exist if *F3′H* outcompeted *DFR-2* for dihydrokaempferol. We hypothesize that *DFR-2* outcompetes *F3′H* at this step suggested by the black "<<<" symbols.

biochemical survey. Although the predominant flavonoids extracted and detected in *L. arvensis* petals were blue and orange anthocyanins, we also found three additional flavonoids representing intermediates and side-branches to the ABP. The flavone luteolin 7-glucoside was previously found in blue petals of *L. arvensis* (Ishikura, 1981) and two flavonols were present in our orange samples: kaempferol 3-glucoside (also identified in Kawashty et al., 1998) and syringetin (this is the first record for compound in *L. arvensis*, although it was previously reported in *L. congestiflora* according to Guo et al., 1998). These compounds are known to have some functions outside of traditional pollinator attraction (Winkel-Shirley, 2002; Jiang et al., 2016). Despite their detection in our UHPLC analysis, they were rare (kaempferol = 1.7% of total flavonoids extracted and the other two were only detected in trace amounts). Beyond these steps, which are very close to the narrow ABP, our biochemical

extraction technique and flavonoid references employed may have limited our ability to detect other flavonoid compounds that may respond to non-pollinator agents of selection.

Our results suggest that flavonoid biosynthetic pathway genes may play additional roles beyond pollinator attraction. The transcriptome and biochemical results clearly indicate that the vast majority of ABP enzymes and biochemicals in petals of *L. arvensis* are primarily focused on anthocyanin production. Whether the anthocyanin compounds in the petals have functions beyond pollinator attraction could not be directly discerned in this study. Previous work by Arista et al. (2013) found that natural populations of blue-flowered *L. arvensis* were more commonly found in the hotter and drier environments of southern Europe in contrast to orange-flowered populations that predominate in the north. Further, their manipulative experiments showed higher fitness for blue-flowered individuals when faced with water stress (Arista et al., 2013). *Lysimachia arvensis* has distinctive cyanidin pigment spots on the undersides of the leaves with unknown function (unpublished data). The predominance of cyanidin anthocyanins in angiosperm vegetative tissues (and lack of pelargonidin-based anthocyanins) regardless of flower color suggests an evolutionary constraint that filters for only tissue-specific changes in floral anthocyanins (Price and Sturgess, 1938; Streisfeld and Rausher, 2011). This correlates with known differences in antioxidant function between malvidin and pelargonidin (blue and orange petals, respectively). The most important determinant of antioxidant activity for anthocyanins is the hydroxylation level of the B-ring (Halbwirth, 2010). Malvidin has three hydroxyl groups whereas pelargonidin has only one and malvidin has much higher antioxidant activity than pelargonidin (Kähkönen and Heinonen, 2003). This may explain why blue-flowered individuals have higher fitness in xeric and high temperatures compared to orange-flowered individuals. Flower life-span is also longer in blue flowers, as every day they open earlier and close latter than orange flowers (Jiménez-López, 2019). Whether this is due to the different anthocyanins in the petals or pleiotropic effects in vegetative tissues is still unknown.

Directional Change in Color From Blue to Orange

The direction of the flower color change in *L. arvensis* remains uncertain although there are clear predictions (Rausher, 2008) and our findings are consistent with these predictions. The directional change from blue to red (and rarely the reverse) is often attributed to the loss of function at a key branchpoint in the ABP (*F3′* and *F3′5′H*) responsible for the blue/purple pigment formation (delphinidin and cyanidin). Without these branches, the precursor, dihydrokaempferol, is shunted directly down the red pathway (pelargonidin; Zufall and Rausher, 2004; Rausher, 2008; Smith and Rausher, 2011). We find a 2.5× decrease in *F3′5′H* expression in orange- compared to blue-flowered samples (coupled with color-differentiating non-synonymous SNPs). In a recent review of the genetic bases for flower color transitions, *F3′5′H* or/and *F3′* were implicated in all 10 blue to red transitions documented from natural systems (see **Supplementary Table 3** in Wheeler and Smith, 2019). Following the inactivation of the delphinidin branch of the ABP and the redirection of flux down the pelargonidin branch, subsequent evolution of substrate specificity by recruiting *DFR-2* (expression ∼600× O > B) instead of the delphinidin-targeted *DFR-1*, would compete for any remaining *F3′* expression to metabolize dihydrokaempferol down the pelargonidin branch (Johnson et al., 2001; Zufall and Rausher, 2004; Smith and Rausher, 2011). Wheeler and Smith (2019) find only two of ten examples from blue to red transitions implicated changes in *DFR*, suggesting that this may be a secondary modification after the initial mutation of large effect (decreased activity of *F3′5′H*). Finally, subsequent changes, such as increased substrate specificity for *BZ1* and other post-translational modifications (e.g., glycosylation) would eventually produce pelargonidin-3-glucoside (**Figure 7**). By coupling tissue-specific expression changes in *F3′5′H* and transitioning from *DFR-1* to *DFR-2*, these evolutionary changes may arrive with very little pleiotropic cost to the organism (Streisfeld and Rausher, 2011), especially if expression changes are restricted to petals via trans-regulatory changes (e.g., *LaMYB61*).

In addition to the predictions based on gene expression and substrate specificity, we found additional evidence consistent with a blue-to-orange transition when examining the *F3′5′H*. In the SNP survey, blue samples were often variable (18/22 = ∼81%) whereas in orange samples, only five SNPs were variable (5/22 = 23%). This pattern is consistent with a selective sweep during the transition from blue to orange causing a reduction in variation in the orange samples (although there are other demographic patterns that could be responsible). Further evidence for a blue-to-orange transition in *L. arvensis* comes from the *Lysimachia* ITS phylogeny indicating that blue is the ancestral flower color for *L. arvensis* and the orange-flowered individuals are the derived state (Jiménez-López, 2019; Jiménez-López et al., in review).

CONCLUSION

The transcriptomic and biochemical analysis of the flavonoid biosynthetic pathway presented in this study provides an important foundation for understanding the molecular genetic basis of flower color polymorphism in *L. arvensis*. The differential expression between flower colors, particularly along the ABP branch, is consistent with the main anthocyanin compounds found in blue and orange petals. As we hypothesized, *F3′5′H* and *DFR* are the principal genes guiding dihydrokaempferol to the proper anthocyanin formation. Our study provides a valuable resource for future molecular studies of *Lysimachia* flowers and sheds light on more general evolutionary processes underlying the diversity of flower color variation.

DATA AVAILABILITY STATEMENT

All raw sequence to the NCBI SRA database under BioProject accession number PRJNA701383. Phylogenetic analyses of

F3'5'H and DFR have been placed on Figshare at doi: 10.6084/m9.figshare.13911287.v1.

AUTHOR CONTRIBUTIONS

MA, PO, EN, JW, and FJ-L conceived and designed the experiments. JW and FJ-L carried out the sampling. FR-C, KR, and BI performed the assembly. KR, BI, and MS-C performed the differential expression analysis. AF developed the UHPLC-MS methods. EN and MS-C analyzed the UHPLC-MS data. MA, PO, EN, JW, FJ-L, and MS-C drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.633979/full#supplementary-material

REFERENCES

Albert, N. W., Davies, K. M., and Schwinn, K. E. (2014). Gene regulation networks generate diverse pigmentation patterns in plants. *Plant Signal Behav*. 9:e29526. doi: 10.4161/psb.29526

Anders, S., McCarthy, D. J., Chen, Y., Okoniewski, M., Smyth, G. K., Huber, W., et al. (2013). Count-based differential expression analysis of RNA sequencing data using R and bioconductor. *Nat. Protoc.* 8, 1765–1786. doi: 10.1038/nprot.2013.099

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc (accessed October 20, 2020).

Arista, M., Talavera, M., Berjano, R., and Ortiz, P. L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.* 101, 1613–1622. doi: 10.1111/1365-2745.12151

Barkman, T. J. (2003). Evidence for positive selection on the floral scent gene isoeugenol-O-methyltransferase. *Mol. Biol. Evol.* 20, 168–172. doi: 10.1093/molbev/msg030

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bomati, E. K., Austin, M. B., Bowman, M. E., Dixon, R. A., and Noel, J. P. (2005). Structural elucidation of Chalcone reductase and implications for deoxychalcone biosynthesis. *Int. J. Biol. Chem.* 280, 30496–30503. doi: 10.1074/jbc.m502239200

Butler, T., Dick, C., Carlson, M. L., and Whittall, J. B. (2014). Transcriptome analysis of a petal anthocyanin polymorphism in the arctic mustard, Parrya nudicaulis. *PLoS One* 9:e101338. doi: 10.1371/journal.pone.0101338

Casimiro-Soriguer, I., Narbona, E., Buide, M. L., del Valle, J. C., and Whittall, J. B. (2016). Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (Caryophyllaceae). *Front. Plant Sci.* 7:204. doi: 10.3389/fpls.2016.00204

Chen, D., Liu, Y., Pan, Q., Li, F., Zhang, Q., Ge, X., et al. (2018). De novo transcriptome assembly, gene expressions and metabolites for flower color variation of two garden species in Brassicaceae. *Sci Hortic.* 240, 592–602. doi: 10.1016/j.scienta.2018.06.057

Clegg, M. T., and Durbin, M. L. (2000). Flower color variation: a model for the experimental study of evolution. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7016–7023. doi: 10.1073/pnas.97.13.7016

Coyne, J. A., and Orr, H. A. (1998). The evolutionary genetics of speciation. *Phil. Trans. Roy. Soc. Lond. B* 353, 287–305. doi: 10.1098/rstb.1998.0210

Dalrymple, R. L., Kemp, D. J., Flores−Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985. doi: 10.1111/nph.16737

Davies, K. M. (2009). "Modifying anthocyanin production in flowers," in *Anthocyanins. Biosynthesis, Functions, and Applications*, eds C. Winefield, K. Davies, and K. Gould (New York, NY: Springer), 49–80. doi: 10.1007/978-0-387-77335-3

Davies, K. M., and Schwinn, K. E. (2006). "Molecular biology and biotechnology of flavonoid biosynthesis," in *Flavonoids. Chemistry, Biochemistry and Applications*, eds ØM. Andersen and K. R. Markham (Boca Raton, FL: CRC Press), 143–218. doi: 10.1201/9781420039443.ch3

Des Marais, D. L., and Rausher, M. D. (2008). Escape from adaptive conflict after duplication in an anthocyanin pathway gene. *Nature* 454, 762–765. doi: 10.1038/nature07092

Dick, C. A., Buenrostro, J., Butler, T., Carlson, M. L., Kliebenstein, D. J., and Whittall, J. B. (2011). Arctic mustard flower color polymorphism controlled by petal-specific downregulation at the threshold of the anthocyanin biosynthetic pathway. *PLoS One* 6:e18230. doi: 10.1371/journal.pone.0018230

Duan, H. R., Wang, L. R., Cui, G. X., Zhou, X. H., Duan, X. R., and Yang, H. S. (2020). Identification of the regulatory networks and hub genes controlling alfalfa floral pigmentation variation using RNA-sequencing analysis. *BMC Plant Biol.* 20:110. doi: 10.1186/s12870-020-2322-9

Esfeld, K., Berardi, A. E., Moser, M., Bossolini, E., Freitas, L., and Kuhlemeier, C. (2018). Pseudogenization and resurrection of a speciation gene. *Curr. Biol.* 28, 3776–3786. doi: 10.1016/j.cub.2018.10.019

Fan, X., Fan, B., Wang, Y., and Yang, W. (2016). Anthocyanin accumulation enhanced in Lc-transgenic cotton under light and increased resistance to bollworm. *Plant Biotechnol. Rep.* 10, 1–11. doi: 10.1007/s11816-015-0382-3

Feng, C., Ding, D., Feng, C., and Kang, M. (2020). The identification of an R2R3-MYB transcription factor involved in regulating anthocyanin biosynthesis in *Primulina swinglei* flowers. *Gene* 752:144788. doi: 10.1016/j.gene.2020.144788

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Ferguson, L. F. (1972). "Anagallis L," in *Flora Europaea*, Vol. 3, eds T. G. Tutin, V. H. Heywood, N. A. Burges, D. H. Valentine, S. M. Walters, and D. A. Webb (Cambridge: Cambridge University Press), 28–29.

Freyre, R., and Griesbach, R. J. (2004). Inheritance of flower color in *Anagallis monelli* L. *HortScience* 39, 1220–1223. doi: 10.21273/HORTSCI.39.6.1220

Freyre, R., Wilson, S. B., Knox, G. W., Uzdevenes, C., Gu, L., and Quesenberry, K. H. (2015). Breeding and genetic studies of *Ruellia simplex* (Mexican petunia). *Acta Hortic.* 1087, 113–120. doi: 10.17660/actahortic.2015.1087.12

Gao, J., Peng, H., Chen, F., Luo, M., and Li, W. (2019). Genome-wide analysis of transcription factors related to anthocyanin biosynthesis in carmine radish (*Raphanus sativus* L.) fleshy roots. *PeerJ* 7:e8041. doi: 10.7717/peerj.8041

Gao, L., Yang, H., Liu, H., Yang, J., and Hu, Y. (2016). Extensive transcriptome changes underlying the flower color intensity variation in *Paeonia ostii*. *Front. Plant Sci.* 6:1205. doi: 10.3389/fpls.2015.01205

Gates, D. J., Olson, B. J. S. C., Clemente, T. E., and Smith, S. D. (2017). A novel R3 MYB transcriptional repressor associated with the loss of floral pigmentation in Iochroma. *New Phytol.* 217, 1346–1356. doi: 10.1111/nph.14830

Gehrig, H. H., Winter, K., Cushman, J., Borland, A., and Taybi, T. (2000). An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. *Plant Mol. Biol. Rep.* 18, 369–376. doi: 10.1007/bf02825065

Gibbs, P. E., and Talavera, S. (2001). Breeding system studies with three species of *Anagallis* (Primulaceae): self-incompatibility and reduced female fertility in *A. monelli* L. *Ann. Bot.* 88, 139–144. doi: 10.1006/anbo.2001.1439

Gigord, L. D. B., Macnair, M. R., and Smithson, A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6253–6255. doi: 10.1073/pnas.111162598

Giordano, D., Provenzano, S., Ferrandino, A., Vitali, M., Pagliarani, C., Roman, F., et al. (2016). Characterization of a multifunctional caffeoyl-CoA O -methyltransferase activated in grape berries upon drought stress. *Plant. Physiol. Biochem.* 101, 23–32. doi: 10.1016/j.plaphy.2016.01.015

Gómez, J. M., Perfectti, F., Armas, C., Narbona, E., González-Megías, A., Navarro, L., et al. (2020). Within-individual phenotypic plasticity in flowers fosters pollination niche shift. *Nat. Commun.* 11:4019. doi: 10.1038/s41467-020-17875-1

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29, 644–652. doi: 10.1038/nbt.1883

Guo, J., Yu, D.-L., Xu, L., Zhu, M., and Yang S.-L. (1998). Flavonol glycosides from Lysimachia congestiflora. *Phytochemistry* 48, 1445–1447. doi: 10.1016/s0031-9422(97)01025-x

Guo, L., Gao, L., Ma, X., Guo, F., Ruan, H., Bao, Y., et al. (2019). Functional analysis of flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylases from tea plant (*Camellia sinensis*), involved in the B-ring hydroxylation of flavonoids. *Gene* 717:144046. doi: 10.1016/j.gene.2019.144046

Haas, B., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., et al. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8, 1494–1512. doi: 10.1038/nprot.2013.084

Halbwirth, H. (2010). The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway. *Int. J. Mol. Sci.* 11, 595–621. doi: 10.3390/ijms11020595

Harborne, J. B. (1968). Comparative biochemistry of the flavonoids. VII. Correlations between flavonoid pigmentation and systematics in the family Primulaceae. *Phytochemistry* 7, 1215–1230. doi: 10.1016/S0031-9422(00)85616-2

Henikoff, S., and Henikoff, J. G. (1992). Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10915–10919. doi: 10.1073/pnas.89.22.10915

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., and Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J. Exp. Bot.* 62, 2465–2483. doi: 10.1093/jxb/erq442

Ho, W. W., and Smith, S. D. (2016). Molecular evolution of anthocyanin pigmentation genes following losses of flower color. *BMC Evol. Biol.* 16:98. doi: 10.1186/s12862-016-0675-3

Holton, T. A., and Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7, 1071–1083. doi: 10.1105/tpc.7.7.1071

Hopkins, R., and Rausher, M. D. (2011). Identification of two genes causing reinforcement in the texas wildflower Phlox drummondii. *Nature* 469, 411–414. doi: 10.1038/nature09641

Huxley, J. S. (1955). Morphism and evolution. *Heredity* 9, 1–51. doi: 10.1038/hdy.1955.1

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator−mediated selection. *Am. Nat.* 165, 225–237. doi: 10.1086/426714

Ishiguro, K., Taniguchi, M., and Tanaka, Y. (2012). Functional analysis of Antirrhinum kelloggii flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase genes; critical role in flower color and evolution in the genus *Antirrhinum*. *J. Plant Res.* 125, 451–456. doi: 10.1007/s10265-011-0455-5

Ishikura, N. (1981). Flavonoids in the petal cells of *Anagallis arvensis* f. coerulea containing a blue crystalline anthocyanin. *Z. Pflanzenphysiol.* 103, 469–473. doi: 10.1016/s0044-328x(81)80170-5

Jiang, N., Doseff, A., and Grotewold, E. (2016). Flavones: from biosynthesis to health benefits. *Plants* 5:27. doi: 10.3390/plants5020027

Jiménez-López, F. J. (2019). *Polimorfismo en el Color Floral y Especiación en Lysimachia Arvensis*. Ph.D. dissertation, University of Seville, Seville.

Jiménez-López, F. J., Matas, L., Arista, M., and Ortiz, P. L. (2019). Flower colour segregation and flower discrimination under the bee vision model in the polymorphic *Lysimachia arvensis*. *Plant Biosyst.* 154, 535–543. doi: 10.1080/11263504.2019.1651776

Jiménez-López, F. J., Ortiz, P. L., Talavera, M., Pannell, J. R., and Arista, M. (2020). The role of lateral and vertical herkogamy in the divergence of the blue- and red-flowered lineages of *Lysimachia arvensis*. *Ann. Bot.* 125, 1127–1135. doi: 10.1093/aob/mcaa047

Jiménez-López, F. J., Viruel, J., Arista, M., Ortiz, P. L., and Talavera, M. (in review). Molecular approaches reveal speciation between red and blue flowered plants in the Mediterranean species of *Lysimachia arvensis* and *Lysimachia monelli* (Primulaceae). *Bot. J. Linn. Soc.*

Johnson, E. T., Ryu, S., Yi, H., Shin, B., Cheong, H., and Choi, G. (2001). Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant J.* 25, 325–333. doi: 10.1046/j.1365-313x.2001.00962.x

Kähkönen, M. P., and Heinonen, M. (2003). Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* 51, 628–633. doi: 10.1021/jf025551i

Kawashty, S., El-Garf, I., and El-Negoumy, S. (1998). Chemosystematics of *Anagallis arvensis* L. (Primulaceae). *Biochem. Syst. Ecol.* 26, 663–668. doi: 10.1016/s0305-1978(98)00008-8

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Kellenberger, R. T., Byers, K. J. R. P., De Brito Francisco, R. M., Staedler, Y. M., LaFountain, A. M., Schönenberger, J., et al. (2019). Emergence of a floral colour polymorphism by pollinator-mediated overdominance. *Nat. Commun.* 10:63. doi: 10.1038/s41467-018-07936-x

Koes, R., Verweij, W., and Quattrocchio, F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* 10, 236–242. doi: 10.1016/j.tplants.2005.03.002

Koski, M. H., MacQueen, D., and Ashman, T.-L. (2020). Floral pigmentation has responded rapidly to global change in ozone and temperature. *Cur. Biol.* 30, 4425–4431. doi: 10.1016/j.cub.2020.08.077

Lai, Y., Li, H., and Yamagishi, M. (2013). A review of target gene specificity of flavonoid R2R3-MYB transcription factors and a discussion of factors contributing to the target gene selectivity. *Front. Biol.* 8:577–598. doi: 10.1007/s11515-013-1281-z

Lang, X., Li, N., Li, L., and Zhang, S. (2019). Integrated metabolome and transcriptome analysis uncovers the role of anthocyanin metabolism in michelia maudiae. *Int. J. Genomics* 2019:4393905. doi: 10.1155/2019/4393905

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Larter, M., Dunbar-Wallis, A., Berardi, A. E., and Smith, S. D. (2018). Convergent evolution at the pathway level: predictable regulatory changes during flower color transitions. *Mol. Biol. Evol.* 35, 2159–2169. doi: 10.1093/molbev/msy117

Lawrence, W. J. C., Price, J. R., Robinson, G. M., and Robinson, R. (1939). The distribution of anthocyanins in flowers, fruits and leaves. *Phil. Trans. Roy. Soc. Lond. B* 230, 149–178. doi: 10.1098/rstb.1939.0006

Le Maitre, N. C., Pirie, M. D., and Bellstedt, D. U. (2019). An approach to determining anthocyanin synthesis enzyme gene expression in an evolutionary context: an example from Erica plukenetii. *Ann. Bot.* 124, 121–130. doi: 10.1093/aob/mcz046

Lee, W. J., Jeong, C. Y., Kwon, J., Van Kien, V., Lee, D., Hong, S. W., et al. (2016). Drastic anthocyanin increase in response to PAP1 overexpression in fls1 knockout mutant confers enhanced osmotic stress tolerance in *Arabidopsis thaliana*. *Plant Cell Rep.* 35, 2369–2379. doi: 10.1007/s00299-016-2040-9

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323

Li, B., Fan, R., Guo, S., Wang, P., Zhu, X., Fan, Y., et al. (2019). The *Arabidopsis* MYB transcription factor, MYB111 modulates salt responses by regulating flavonoid biosynthesis. *Environ. Exp. Bot.* 166:103807. doi: 10.1016/j.envexpbot.2019.103807

Li, L., Zhai, Y., Luo, X., Zhang, Y., and Shi, Q. Q. (2019). Comparative transcriptome analyses reveal genes related to pigmentation in the petals of red and white *Primula vulgaris* cultivars. *Physiol. Mol. Biol. Plants* 25, 1029–1041. doi: 10.1007/s12298-019-00664-6

Li, S. (2014). Transcriptional control of flavonoid biosynthesis. *Plant Signal Behav.* 9:e27522. doi: 10.4161/psb.27522

Liu, Q., Luo, L., and Zheng, L. (2018). Lignins: biosynthesis and biological functions in plants. *Int. J. Mol. Sci.* 19:335. doi: 10.3390/ijms19020335

Lou, Q., Liu, Y., Qi, Y., Jiao, S., Tian, F., Jiang, L., et al. (2014). Transcriptome sequencing and metabolite analysis reveals the role of delphinidin metabolism in flower colour in grape hyacinth. *J. Exp. Bot.* 65, 3157–3164. doi: 10.1093/jxb/eru168

Lulin, H., Xiao, Y., Pei, S., Wen, T., and Shangqin, H. (2012). The first Illumina-based de novo transcriptome sequencing and analysis of safflower flowers. *PLoS One* 7:e38653. doi: 10.1371/journal.pone.0038653

Martin, J. A., and Wang, Z. (2011). Next-generation transcriptome assembly. *Nat. Rev. Genet.* 12, 671–682. doi: 10.1038/nrg3068

McCarthy, E. W., Berardi, A. E., Smith, S. D., and Litt, A. (2017). Related allopolyploids display distinct floral pigment profiles and transgressive pigments. *Am. J. Bot.* 104, 92–101. doi: 10.3732/ajb.1600350

Moneim, A. I, Atta, A., and Shehata, A. A. (2003). On the delimitation of *Anagallis arvensis* L. (Primulaceae) 1. Evidence based on micromorphological characters, palynological features and karyological studies. *Pak. J. Biol. Sci.* 6, 29–35. doi: 10.3923/pjbs.2003.29.35

Morita, Y., Hoshino, A., Kikuchi, Y., Okuhara, H., Ono, E., Tanaka, Y., et al. (2005). Japanese morning glory dusky mutants displaying reddish-brown or purplish-gray flowers are deficient in a novel glycosylation enzyme for anthocyanin biosynthesis, UDP-glucose:anthocyanidin 3-O-glucoside-2"-O-glucosyltransferase, due to 4-bp insertions in. *Plant J.* 42, 353–363. doi: 10.1111/j.1365-313x.2005.02383.x

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Ng, J., Freitas, L. B., and Smith, S. D. (2018). Stepwise evolution of floral pigmentation predicted by biochemical pathway structure. *Evolution* 72:13589. doi: 10.1111/evo.13589

Nguyen, N. H., Kim, J. H., Kwon, J., Jeong, C. Y., Lee, W., Lee, D., et al. (2016). Characterization of *Arabidopsis thaliana* flavonol synthase 1 (FLS1) - overexpression plants in response to abiotic stress. *Plant Physiol. Biochem.* 103, 133–142. doi: 10.1016/j.plaphy.2016.03.010

Ortiz, P. L., Berjano, R., Talavera, M., Rodríguez-Zayas, L., and Arista, M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: how is the red morph maintained in Mediterranean environments? *Perspect. Plant Ecol. Evol. Syst.* 17, 142–150. doi: 10.1016/j.ppees.2015.01.004

Ortiz-Barrientos, D. (2013). The color genes of speciation in plants. *Genetics* 194, 39–42. doi: 10.1534/genetics.113.150466

Petit, P., Granier, T., d' Estaintot, B. L., Manigand, C., Bathany, K., Schmitter, J. M., et al. (2007). Crystal structure of grape dihydroflavonol 4-reductase, a key enzyme in flavonoid biosynthesis. *J. Mol. Biol.* 368, 1345–1357. doi: 10.1016/j.jmb.2007.02.088

Price, J. R., and Sturgess, V. C. (1938). A survey of anthocyanins. *VI. Biochem. J.* 32, 1658–1660. doi: 10.1042/bj0321658

Pujadas, A. (1997). "Anagallis," in *Flora Ibérica*, Vol. V, eds S. Castroviejo, S. Aedo, M. Laínz, R. Morales, F. M. Garmendia, and G. N. Feliner (Madrid: C.S.I.C).

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Quattrocchio, F., Baudry, A., Lepiniec, L., and Grotewold, E. (2006). "The regulation of flavonoid biosynthesis," in *The Science of Flavonoids*, ed. E. Grotewold (New York, NY: Springer-Verlag), 97–122. doi: 10.1007/978-0-387-28822-2_4

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Rausher, M. D., and Simms, E. L. (1989). The evolution of resistance to herbivory in Ipomoea purpurea . I. Attempts to detect selection. *Evolution* 43, 563–572. doi: 10.1111/j.1558-5646.1989.tb04252.x

Reiseberg, L. H., and Blackman, B. K. (2010). Speciation genes in plants. *Ann. Bot.* 106, 439–455. doi: 10.1093/aob/mcq126

Roberts, W. R., and Roalson, E. H. (2017). Comparative transcriptome analyses of flower development in four species of *Achimenes* (Gesneriaceae). *BMC Genomics* 18:240. doi: 10.1186/s12864-017-3623-8

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2009). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Robinson, M. D., and Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* 11:R25. doi: 10.1186/gb-2010-11-3-r25

Ruegger, M., Meyer, K., Cusumano, J. C., and Chapple, C. (1999). Regulation of Ferulate-5-hydroxylase expression in *Arabidopsis* in the context of sinapate ester biosynthesis. *Plant Physiol.* 119, 101–110. doi: 10.1104/pp.119.1.101

Saito, K., Yonekura-Sakakibara, K., Nakabayashi, R., Higashi, Y., Yamazaki, M., Tohge, T., et al. (2013). The flavonoid biosynthetic pathway in *Arabidopsis*: structural and genetic diversity. *Plant Physiol. Biochem.* 72, 21–34. doi: 10.1016/j.plaphy.2013.02.001

Schemske, D. W., and Bierzychudek, P. (2007). Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was wright right? *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Seitz, C., Ameres, S., and Forkmann, G. (2007). Identification of the molecular basis for the functional difference between flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase. *FEBS Lett.* 581, 3429–3434. doi: 10.1016/j.febslet.2007.06.045

Shimada, N., Sasaki, R., Sato, S., Kaneko, T., Tabata, S., Aoki, T., et al. (2005). A comprehensive analysis of six dihydroflavonol 4-reductases encoded by a gene cluster of the *Lotus japonicus* genome. *J. Exp. Bot.* 56, 2573–2585. doi: 10.1093/jxb/eri251

Shipunov, A., Kosenko, Y., and Volkova, P. (2011). Floral polymorphism in common primrose (Primula vulgaris Huds., Primulaceae) of the Northeastern Black Sea coast. *Plant Syst. Evol.* 296, 167–178. doi: 10.1007/s00606-011-0484-5

Smith, S. D., and Rausher, M. D. (2011). Gene loss and parallel evolution contribute to species difference in flower color. *Mol. Biol. Evol.* 28, 2799–2810. doi: 10.1093/molbev/msr109

Smith, S. D., Wang, S., and Rausher, M. D. (2012). Functional evolution of an anthocyanin pathway enzyme during a flower color transition. *Mol. Biol. Evol.* 30, 602–612. doi: 10.1093/molbev/mss255

Sobel, J. M., and Streisfeld, M. A. (2013). Flower color as a model system for studies of plant evo-devo. *Front. Plant Sci.* 4:321. doi: 10.3389/fpls.2013.00321

Song, L., and Florea, L. (2015). Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience* 4, 48. doi: 10.1186/s13742-015-0089-y

Stracke, R., Werber, M., and Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol.* 4, 447–456. doi: 10.1016/s1369-5266(00)00199-0

Strauss, S., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press), 120–138.

Streisfeld, M. A., and Rausher, M. D. (2009). Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species. *New Phytol.* 183, 751–763. doi: 10.1111/j.1469-8137.2009.02929.x

Streisfeld, M. A., and Rausher, M. D. (2011). Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* 65, 629–642. doi: 10.1111/j.1558-5646.2010.01165.x

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313x.2008.03447.x

Tripp, E. A., and Manos, P. S. (2008). Is floral specialization an evolutionary dead-end pollination system transitions in *Ruellia* (Acanthaceae). *Evolution* 62, 1712–1737. doi: 10.1111/j.1558-5646.2008.00398.x

Van der Kooi, C. J., Kevan, P. G., and Koski, M. H. (2019). The thermal ecology of flowers. *Ann. Bot.* 124, 343–353.

Van der Niet, T., Peakall, R., and Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Ann. Bot.* 113, 199–212. doi: 10.1093/aob/mct290

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Wessinger, C. A., and Rausher, M. D. (2012). Lessons from flower colour evolution on targets of selection. *J. Exp. Bot.* 63, 5741–5749. doi: 10.1093/jxb/ers267

Wessinger, C. A., and Rausher, M. D. (2013). Predictability and irreversibility of genetic changes associated with flower color evolution in *Penstemon barbatus*. *Evolution* 68, 1058–1070. doi: 10.1111/evo.12340

Wessinger, C. A., and Rausher, M. D. (2014). Ecological transition predictably associated with gene degeneration. *Mol. Biol. Evol.* 32, 347–354. doi: 10.1093/molbev/msu298

Wheeler, L. C., and Smith, S. D. (2019). Computational modeling of anthocyanin pathway evolution: biases, hotspots, and trade-offs. *Integr. Comp. Biol.* 59, 585–598. doi: 10.1093/icb/icz049

Widmer, A., Lexer, C., and Cozzolino, S. (2009). Evolution of reproductive isolation in plants. *Heredity* 102, 31–38. doi: 10.1038/hdy.2008.69

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.* 5, 218–223. doi: 10.1016/s1369-5266(02)00256-x

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biol.* 20, 257. doi: 10.1186/s13059-019-1891-0

Xu, W., Dubos, C., and Lepiniec, L. (2015). Transcriptional control of flavonoid biosynthesis by MYB–bHLH–WDR complexes. *Trends Plant Sci.* 20, 176–185. doi: 10.1016/j.tplants.2014.12.00

Yuan, Y. W., Byers, K. J., and Bradshaw, H. (2013). The genetic control of flower–pollinator specificity. *Curr. Opin. Plant Biol.* 16, 422–428. doi: 10.1016/j.pbi.2013.05.004

Zhang, Y., Zhou, T., Dai, Z., Dai, X., Li, W., Cao, M., et al. (2020). Comparative transcriptomics provides insight into floral color polymorphism in a *Pleione limprichtii* orchid population. *Int. J. Mol. Sci.* 21:247. doi: 10.3390/ijms21010247

Zhao, L., Gao, L., Wang, H., Chen, X., Wang, Y., Yang, H., et al. (2013). The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Funct. Integr. Genomics* 13, 75–98. doi: 10.1007/s10142-012-0301-4

Zufall, R. A., and Rausher, M. D. (2004). Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* 428, 847–850. doi: 10.1038/nature02489

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Transgenerational Plasticity in Flower Color Induced by Caterpillars

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Variation in flower color due to transgenerational plasticity could stem directly from abiotic or biotic environmental conditions. Finding a link between biotic ecological interactions across generations and plasticity in flower color would indicate that transgenerational effects of ecological interactions, such as herbivory, might be involved in flower color evolution. We conducted controlled experiments across four generations of wild radish (*Raphanus sativus*, Brassicaceae) plants to explore whether flower color is influenced by herbivory, and to determine whether flower color is associated with transgenerational chromatin modifications. We found transgenerational effects of herbivory on flower color, partly related to chromatin modifications. Given the presence of herbivory in plant populations worldwide, our results are of broad significance and contribute to our understanding of flower color evolution.

Keywords: flower color, methylation, transgenerational plasticity, herbivory, anthocyanins, chromatin modifications, Brassicaceae

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INTRODUCTION

Flower color is a key trait in angiosperm evolution. It is one of the most conspicuous traits under natural selection because of its potential role in attracting pollinators, and the subsequent influence on reproductive fitness (Spaethe et al., 2001; Wester and Lunau, 2017). Flower color is commonly related to quality or quantity of floral rewards, such as nectar or pollen (Fenster et al., 2004; Rausher, 2008). This relationship opens up the possibility of evolutionary diversification driven by pollinators acting on flower color variation within a species (Gegear and Burns, 2007; Losada et al., 2015; Sobral et al., 2015; Narbona et al., 2018).

Color in flowers is mainly due to the presence of pigments (Van Der Kooi et al., 2019). Among plant pigments, flavonoids—specifically anthocyanins—are the most common and diverse type (Tanaka et al., 2008). However, besides pollinator attraction, anthocyanins, and other groups of flavonoids that share a common biosynthetic pathway, may act as deterrents or defensive compounds against floral antagonists (Lev-Yadun and Gould, 2008; Zhang et al., 2013). Thus, herbivores, seed predators, and pathogens could also play an important role as non-pollinator agents of selection in flower color (Strauss and Whittall, 2006; Veiga et al., 2015). In addition, abiotic factors may also shape flower color variation (Dalrymple et al., 2020).

Flower color polymorphism within a species occurs when more than one flower color morph is present among individuals in the same or different populations (Warren and Mackenzie, 2001; Narbona et al., 2018). Causes of flower color polymorphism include different selection pressures

imposed by mutualistic pollinators, antagonistic herbivores, or pathogens (Veiga et al., 2015; Narbona et al., 2018). This process is supposed to require the establishment and maintenance of a flower color mutant genotype in the population (Sobel et al., 2010; Del Valle et al., 2019) with the flower color variation based on the downregulation of structural or regulatory genes of the biosynthetic pathway of pigments accumulated in flowers (Sobel and Streisfeld, 2013; Roberts and Roalson, 2017). Thus, flower color polymorphism is expected to involve changes in nucleotide sequence that are transmitted to subsequent generations. Recently, it has been found that a radical change in flower morphology, including petal color, in *Moricandia arvensis* is due to within-individual plasticity produced by seasonal changes in climatic conditions (Gómez et al., 2020). A transcriptomic analysis found a coordinated response of more than 600 genes that was differentially expressed between two types of flowers suggesting a genetic basis for this plastic response (Gómez et al., 2020; see also Laitinen and Nikoloski, 2019).

As flower color is a trait highly affected by evolutionary constraints due to the influence of pollinator and non-pollinator agents of selection (Schiestl and Johnson, 2013; Sletvold et al., 2016), plasticity in flower color is expected to be low in comparison with other floral and vegetative traits (Del Valle et al., 2018, 2019; but see Gómez et al., 2020). However, flower color may show some degree of plasticity in response to abiotic as well as to biotic stressors, such as herbivores or pathogens (Wang et al., 2017; Rusman et al., 2019b; Koski and Galloway, 2020). Beyond this within-generational plasticity, it is not yet known whether there are possible transgenerational plastic effects of herbivory on flower color. In other words, we do not know if flower color plasticity is influenced by herbivory events across generations. Exploring whether the ecological experiences of previous generations, such as herbivory, influence flower color may contribute to our understanding of flower color evolution.

Transgenerational phenotypic plasticity challenges our current knowledge of evolutionary processes. Environmental factors can modify phenotypes directly via epigenetic modifications (Jablonka and Raz, 2009) that are transmissible across generations and can be adaptive (Jablonka and Raz, 2009; Herman and Sultan, 2016). Such phenotypic modifications can be manifested in a variety of ecologically relevant traits (Bossdorf et al., 2010), including the induction of defenses against herbivores (Herrera and Bazaga, 2013), and with subsequent effects on plant fitness (Herrera et al., 2014), at least partially independent of genetic variation (Verhoeven et al., 2010). Natural selection could act on this variation through its effect on ecological interactions that in turn affect fitness (Bossdorf et al., 2008; Alonso et al., 2019). In this way, transgenerational plasticity can influence the course of evolution (Bonduriansky and Day, 2009). Despite recent discoveries about the ecological and evolutionary implications of transgenerational effects (Verhoeven et al., 2016; Colicchio and Herman, 2020), transgenerational consequences of herbivory on flower color have not yet, to our knowledge, been explored.

We conducted controlled experiments with the wild radish (*Raphanus sativus* L.) across four generations. This annual species shows a petal color polymorphism (**Figure 1**, white, pink, yellow, and bronze) due to the independent absence and presence of anthocyanins and carotenoids (Strauss et al., 2004; Strauss and Whittall, 2006). Here, we explore how the presence of petal anthocyanins across generations depends on herbivory by caterpillars of a specialized herbivore and whether such phenotypic changes are associated with chromatin modifications (i.e., genome-wide methylation).

MATERIALS AND METHODS

Study Organisms

In California, wild radish, *Raphanus sativus* L. (Brassicaceae) is a naturalized, self-incompatible, herbaceous annual weed that grows along coastal sites, roadsides, and agricultural fields (Campbell and Snow, 2009). Wild radish has a flower color polymorphism that is driven by the presence or absence of anthocyanins (A) and carotenoids (C), with individuals showing either bronze (A+, C+), purple (A+, C−), yellow (A−, C+), or white (A−, C−) flowers (Irwin and Strauss, 2005). The inheritance of the pigments is Mendelian with two independently assorting loci coding for presence of anthocyanin and carotenoids (Irwin and Strauss, 2005).

Seeds germinate in October through November, and plants bloom in March for a period of 3–4 months. A wide variety of herbivores (aphids, snails, slugs, flea beetles, caterpillars, rabbits, and deer) feed on *R. sativus*. Prominent among these herbivores is the larval stage of the white cabbage butterfly, *Pieris rapae*, which was used for the herbivory treatments in this study. White cabbage butterfly caterpillars are specific herbivores to the Brassicaceae family.

Experimental Design

We raised four generations of offspring from a single maternal family displaying white flowers collected from the wild in California. One maternal family (P) was raised in the greenhouse where they experienced no herbivory. After that, three generations (F_1, F_2, and F_3) were raised. The plants used in this study are all descended from half siblings. To test our hypothesis, it was important to control for genetic variation so that any potential epigenetic or plastic mechanisms found could be interpreted and, at least partially, treated independently from genetic variation.

For all generations, we subjected plants to one of two treatments: herbivory or control (non-herbivory). We used a fully crossed, factorial experimental design, where the first factor was the exposure to herbivory in the F_1 generation, the second was exposure in the F_2 generation, and a third was exposure to herbivory in the F_3 generation. In this way our F_3 generation consisted of 130 plants in eight groups (between 12 and 22 plants per group) 68 of them with mothers attacked by herbivores and 62 of them with mothers not exposed to herbivores. This design enabled us to study the effect of herbivory across generations on subsequent flower color.

Plants were grown in germination flats in a greenhouse at the Stanford University Greenhouse Facility. We used a soil mix of 5% fine white sand, 20% potting soil and 75% peat moss



FIGURE 1 | *Raphanus sativus* plants showing flower color polymorphism. **(A)** purple-flowered plants with anthocyanins present in petals. **(B)** White-flowered plants lacking anthocyanin in petals.

(Orchard Supply Hardware, San Jose, CA, United States). Two weeks after germination, seedlings were transferred to 0.8-L pots. Plants were watered once a day and maintained at a 26°C/21°C, under a 12 h/12 h cycle. At the beginning of the experiment, the plants had two leaves. The caterpillars used were all second or third instar. The experiment lasted 2 weeks, until larvae started pupation. The amount of leaf tissue eaten was about 60% of the total leaf area on average but ranged from 40 to 80%. The caterpillars heavily attacked the plants and the plants quickly grew new leaf tissue in response. No plants were killed by the effects of herbivory. The caterpillars were purchased and shipped from Carolina Biological Company (CBC) and were delivered in small plastic containers with five to six larvae per container, and an agar and wheat-based food medium provided *ad libitum*. Caterpillars arrived during their second or third instar and were used in the experiments immediately upon arrival. Two *Pieris rapae* caterpillars were placed on the leaves of each plant in the herbivory treatment and allowed to roam around the plant freely for 2 weeks.

The herbivory treatment lasted for about the first quarter of the plants' life. Plants germinated after 4 or 5 days of sowing the seeds, they flowered around 45 days later, and ended life with mature seeds at 2 months. Pollination was performed by hand with makeup brushes. In each generation, plants from the same treatment were crossed. We collected pollen with the brush from all plants per group and we pollinated all plants within the same group. For the F_3 generation, four groups with 24 to 44 plants per group depending on the F_1 and F_2 treatment combination, were used. The goal was to pollinate all flowers on each plant. Several seeds per plant were sown and when all plants had germinated seeds, one seedling from each mother was selected using seedlings with similar sizes for all plants. We did not observe any plants with bronze flowers, and yellow forms were rare; thus, we focused on the production of anthocyanins (and we did not study carotenoid plasticity). Petal color of individuals was visually recorded as purple or white (i.e., presence or absence of petal anthocyanins, respectively; **Figure 1**). Biochemical analysis of white petals of *R. sativus* performed with HPLC-DAD showed absence of anthocyanins (E. Narbona et al. unpublished results).

Chromatin Analyses

We collected leaf material from F_2 generation plants before and after treatment. Plants were harvested at approximately 1 week of age before the treatment, and again at 3 weeks of age after the treatment. Plants had two or three leaves in the first sampling and between 5 and 10 leaves in the second sampling. Only fresh leaves that had recently expanded were sampled. We cut out one 2.7 cm diameter disc with a cork borer from a leaf, and leaf material was kept in dry silica gel until DNA extraction. The epigenetic characterization of the individuals before and after the treatment was made by means of methylation sensitive amplification fragment length polymorphism. In this technique, genomic DNA is digested by methylation-sensitive enzymes providing an epigenetic fingerprint for the plants.

We were interested in detecting DNA methylation events experienced by individual genotypes (and not on methylation differences between genotypes). Thus, a simplified MSAP method was performed using only primer combinations with the methylation-sensitive *Hpa*II. *Hpa*II cleaves CCGG sequences when cytosines are not methylated. Cleaving may be impaired when at least one cytosine is hemi-methylated and this process is inactive if one (or both) of the cytosines are fully methylated (Herrera et al., 2012). Thus, within the same genotype, polymorphism of MSAP markers reflects variation

in the methylation status of the CCGG sites. The change from presence to absence implies a methylation event in a locus, and the change from absence to presence implies a de-methylation event (see Herrera et al., 2012 for an application of this simplified MSAP method).

DNA was isolated using the hexadecyl-trimethyl-ammonium-bromide procedure. After that, MSAFLP fingerprinting was performed. We first performed a pilot study with 15 individuals (30 samples) and 6 primer combinations. Based on this approach, we analyzed 106 samples of 53 plants belonging to the F_2 generation using 2 primer combinations. The selected combinations X-AC/M-AC and X-AC/M-ATC were used for fragment amplification. MS-AFLP analyses were carried out by Keygene Laboratories (Netherlands).

Statistical Analyses

Transgenerational Effects of Herbivory on Flower Color

A generalized linear model (binomial error distribution, link logit) for the 130 plants from F_3 was performed to relate the anthocyanin presence (with or without anthocyanin) to the herbivory treatment in F_1, F_2, and F_3 generations. We additionally tested this model including two- and three-way interactions among generation treatments. An alternative model was tested in which the fixed factors included the number of herbivory events across generations and the order of the herbivory treatments across generations (nested in number of herbivory events). All models were compared by means of AICc criterion and the model which presented the best fit included exclusively the herbivory treatment in each generation.

Transgenerational Effects of Maternal Methylation on Flower Color

Only fragments >300 base pairs in size were considered to reduce the potential impact of size homoplasy (Vekemans et al., 2002). Methylation and de-methylation cannot occur at the same time. Therefore, we considered non-methylation events only when de-methylation did not occur on the marker. MSAP marker scores for samples were transformed by comparing with the corresponding values (i.e., same plant individual before the treatment). MSAP marker scores involving a change from 1 to 0 denoted a methylation event of the marker involved. A new sample ($N = 53$ plant individuals) by marker ($N = 402$) score matrix was obtained where each element denoted whether the sample involved had experienced a methylation event (score = 1) or not (score = 0) in the corresponding marker (or missing when a de-methylation event occurred or when loci were already methylated before the treatment and therefore a methylation event was not possible). We assessed the genome-wide methylation per individual as the percentage of methylation events occurring per individual during treatment.

To understand the potential mechanism of transgenerational plasticity in flower color, we analyzed how methylation in F_2 plants affected flower color in the F_3 generation. A generalized linear model (binomial error distribution, link logit) for the response variable anthocyanin presence or absence (1/0) in the F_3 generation was performed to relate this pigment presence to the global methylation of their mothers during treatments (fixed covariate included in the model). We tested alternative models in which herbivory treatments across generations were also included either independently or as the number of inductions in the ancestry line and we selected the model in which only the covariate methylation was included based on the AICc criterion.

Statistical analyses were performed in RStudio for R version 4.0.2 (R Core Team, 2013). Models were fitted using the function glm from the lme4 package (Bates et al., 2015). Statistical significance of fixed effects was determined by analysis of deviance, Type II Wald chi-square tests using the ANOVA function from the car package and the function AICc from the AICcmodavg package.

RESULTS

Transgenerational Effects of Herbivory on Flower Color

In F_3 plants, 94 individuals had white flowers (without anthocyanins) and 36 individuals had pink/purple flowers (with anthocyanins). The presence/absence of anthocyanins in these 130 (F_3) individuals was related to the maternal herbivory treatment (**Table 1**). Plants were more likely to present purple flowers (with anthocyanins) when their mothers had suffered herbivory. The probability of producing petal anthocyanins was around 16% in plants coming from mothers who experienced no herbivory whereas it was around 38% in plants whose mothers were attacked by herbivores (**Table 1** and **Figure 2**). Current and grandmaternal herbivory were not related to flower color and the interactions between generation treatments were not retained in the model (and non-significant).

Transgenerational Effects of Maternal Methylation on Flower Color

The occurrence of methylation during the 2-week herbivory/non-herbivory treatment ranged from 5 to 14% of genome wide loci. The presence of anthocyanin in plants from the F_3 generation was related to the percent genome-wide methylation of their mothers (Estimate 61.069, s.e. 21.127, $Z = 2.891$, $P = 0.004$). Global genome-wide cytosine methylation of F_2 plants was positively related to the probability anthocyanins presence in their offspring (**Figure 3**). The model which included herbivory across generations and methylation in F_2 performed worst in terms of AICc score, thus it is not presented. When including simultaneously both F_2 methylation and maternal herbivory, the latter was not significant.

DISCUSSION

Within-generational plasticity in flower color as well as the role of epigenetic inheritance in flower color variation have been recently reported (Gómez et al., 2020; Han et al., 2020). However, little work has focused on the possible transgenerational nature of plasticity in flower color and its potential eco-evolutionary causes. Here, we show that flower color may be linked to the herbivory, and methylation, in the previous generation. Flower

TABLE 1 | Results of the generalized linear model analyzing flower color (probability of F_3 individuals containing anthocyanin in their petals) as a function of the herbivory in F_1, F_2, and F_3 generations.

Response variable		Estimate	s.e.	*z*-value	*P*-value
	Current herbivory	−0.329	0.407	−0.809	0.418
Flower color in F3	**Maternal herbivory**	−1.139	0.4316	−2.641	**0.008**
(anthocyanin presence)	Grandmaternal herbivory	0.235	0.421	0.558	0.577

Significant P-values are highlighted in bold.

color is not only a trait affected by the biotic and abiotic environment through natural selection and plasticity (Rusman et al., 2019a; Dalrymple et al., 2020), but our results indicate that flower color variation, at least in some cases, may be related to herbivory induced transgenerational plasticity.

Our results also indicate that this transgenerational effect of herbivory on flower color in *R. sativus* may be related to chromatin modifications. Although there are some reports of epigenetic inheritance involved in flower color modification in ornamental plants (Deng et al., 2015; Morita and Hoshino, 2018), this phenomenon has remained virtually unknown in wild plants, with some exceptional, related instances. For example, it has been recently proposed that epigenetic regulation of anthocyanin production may take place under stress-induced conditions in leaves of *Arabidopsis* and poplar (Fan et al., 2018; Cai et al., 2019). The regulation of the expression of a set of anthocyanin biosynthetic genes is mediated thorough the SWR1 ATP-dependent chromatin remodeling complex (Cai et al., 2019). The anthocyanin biosynthetic pathway in floral and foliar tissues shares the same structural genes, and differs only in the identity of transcriptional regulators (Hichri et al., 2011; Albert et al., 2014), opening up the possibility that a similar epigenetic process to that found in *Arabidopsis* and poplar could be acting in *R. sativus*. In fact, changes in methylation levels in an anthocyanin biosynthesis transcription factor have been recently found in petals of *Malus halliana* during flower color fading (Han et al., 2020).

Herbivory had previously been shown to relate to flower coloration, but this result was interpreted as defense traits pleiotropically linked to flower color morph because of the relationship between glucosinolates and the anthocyanin biosynthetic pathway (Strauss et al., 2004; Strauss and Whittall, 2006). Here we find that this relationship may be due to anthocyanins and flower color being plastic themselves, and responding to the same stimulus as plant defenses, expanding our previous understanding of this process. A possible interpretation of our results is that glucosinolates are upregulated in a transgenerational way, and anthocyanins are therefore indirectly induced by herbivory. In addition, anthocyanins share biosynthetic pathway with other flavonoids with well-known functions in plant defense, such as isoflavonoids, flavones, flavonols, and proanthocyanidins (Gould and Lister, 2006). This opens up the possibility that an upregulation of plant defense flavonoids in flowers may pleiotropically increase anthocyanin production due to their common biosynthetic origin (Johnson et al., 2015).

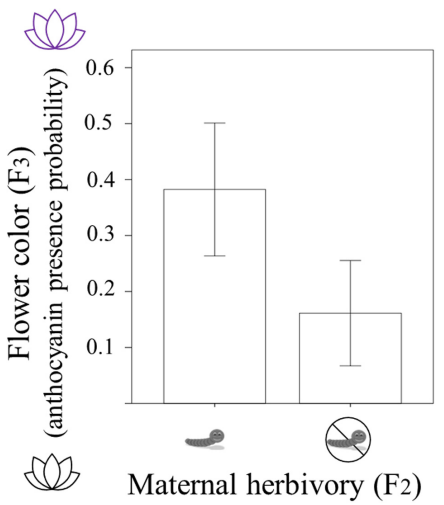


FIGURE 2 | Flower color of *Raphanus sativus* F_3 plants (with white flowers having no anthocyanins and purple flowers presenting anthocyanin; y-axis) depending on whether their mothers experienced herbivory by caterpillars (maternal herbivory in F_2; x-axis). Difference is statistically significant ($P < 0.01$) and bars correspond to two standard errors.

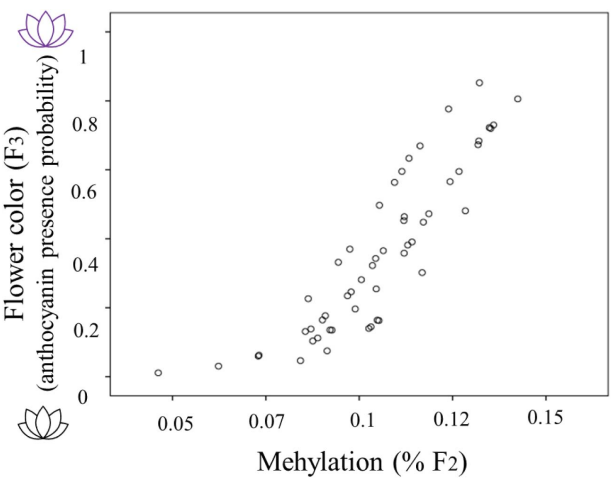


FIGURE 3 | The relationship between flower color of *Raphanus sativus* F_3 plants (purple flowers producing anthocyanins and white flowers lacking anthocyanins) with the genome wide percent methylation in their F_2 mothers. Flower color is estimated as the predicted probability of anthocyanin presence from the saturated model including herbivory treatments and methylation (see "Statistical Analyses" section for details).

The ecological significance of the increase in probability to produce purple flowers lies in the complex pattern of mutualistic and antagonistic interactions this species shows. For example, pollinators, primarily honeybees, prefer to visit flowers of *R. sativus* with an absence of anthocyanin (white and yellow) (Stanton, 1987; Irwin and Strauss, 2005). Additionally, it has been found that herbivore damage in *R. sativus* can induce resistance to florivores in petals. Florivores prefer petals from plants which have not been previously attacked, but this difference is only noticeable in anthocyanin free plants (McCall et al., 2018). We have previously reported (Neylan et al., 2018) that the number of herbivory events across generations affected plant palatability to generalist slugs, but not to specialized caterpillars. Flower color variation seems therefore to be related to transgenerational effects of herbivory and plant and flower palatability (McCall et al., 2018; Neylan et al., 2018). The ecological implications of these multi-generational, multi-faceted effects may affect not only the response-inducing antagonists (herbivores) but mutualists as well, with complex and far-reaching consequences at both the population and community level (Jacobsen and Raguso, 2018; Rusman et al., 2019b; Kessler and Chautá, 2020).

The consequences of transgenerational plasticity through shared pathways and potential pleiotropies with traits that affect reproductive capabilities in offspring, remain understudied but potentially significant (Lehto and Tinghitella, 2020). Our work suggests transgenerational effects of herbivory, related to epigenetic modifications, on flower color variation within a species, improving our understanding of flower color evolutionary processes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MS and RD designed the study. MS and IN analyzed the data and performed the experimental work. MS and EN wrote the manuscript. All authors contributed to the revisions.

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REFERENCES

Albert, N. W., Davies, K. M., Lewis, D. H., Zhang, H., Montefiori, M., Brendolise, C., et al. (2014). A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in Eudicots. *Plant Cell* 26, 962–980. doi: 10.1105/tpc.113.122069

Alonso, C., Ramos-Cruz, D., and Becker, C. (2019). The role of plant epigenetics in biotic interactions. *New Phytol.* 221, 731–737. doi: 10.1111/nph.15408

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bonduriansky, R., and Day, T. (2009). Nongenetic inheritance and its evolutionary implications. *Annu. Rev. Ecol. Evol. Syst.* 40, 103–125. doi: 10.1146/annurev.ecolsys.39.110707.173441

Bossdorf, O., Arcuri, D., Richards, C. L., and Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. *Evol. Ecol.* 24, 541–553. doi: 10.1007/s10682-010-9372-7

Bossdorf, O., Richards, C. L., and Pigliucci, M. (2008). Epigenetics for ecologists. *Ecol. Lett.* 11, 106–115. doi: 10.1111/j.1461-0248.2007.01130.x

Cai, H., Zhang, M., Chai, M., He, Q., Huang, X., Zhao, L., et al. (2019). Epigenetic regulation of anthocyanin biosynthesis by an antagonistic interaction between H2A.Z and H3K4me3. *New Phytol.* 221, 295–308. doi: 10.1111/nph.15306

Campbell, L. G., and Snow, A. A. (2009). Can feral weeds evolve from cultivated radish (*Raphanus sativus*, Brassicaceae)?. *Am. J. Bot.* 96, 498–506. doi: 10.3732/ajb.0800054

Colicchio, J. M., and Herman, J. (2020). Empirical patterns of environmental variation favor adaptive transgenerational plasticity. *Ecol. Evol.* 10, 1648–1665. doi: 10.1002/ece3.6022

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytol.* 228, 1972–1985.

Del Valle, J. C., Alcalde-Eon, C., Escribano-Bailón, M. T., Buide, M. L., Whittall, J. B., and Narbona, E. (2019). Stability of petal color polymorphism: the significance of anthocyanin accumulation in photosynthetic tissues. *BMC Plant Biol.* 19:496. doi: 10.1186/s12870-019-2082-6

Del Valle, J. C., Buide, M. L., Whittall, J. B., and Narbona, E. (2018). Phenotypic plasticity in light-induced flavonoids varies among tissues in *Silene littorea* (Caryophyllaceae). *Environ. Exp. Bot.* 153, 100–107. doi: 10.1016/j.envexpbot.2018.05.014

Deng, J., Fu, Z., Chen, S., Damaris, R. N., Wang, K., Li, T., et al. (2015). Proteomic and epigenetic analyses of lotus (*Nelumbo nucifera*) petals between red and white cultivars. *Plant Cell Physiol.* 56, 1546–1555.

Fan, D., Wang, X., Tang, X., Ye, X., Ren, S., Wang, D., et al. (2018). Histone H3K9 demethylase JMJ25 epigenetically modulates anthocyanin biosynthesis in poplar. *Plant J.* 96, 1121–1136. doi: 10.1111/tpj.14092

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* 35, 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Gegear, R. J., and Burns, J. G. (2007). The birds, the bees, and the virtual flowers: Can pollinator behavior drive ecological speciation in flowering plants? *Am. Nat.* 170, 551–566. doi: 10.1086/521230

Gómez, J. M., Perfectti, F., Armas, C., Narbona, E., González-Megías, A., Navarro, L., et al. (2020). Within-individual phenotypic plasticity in flowers fosters pollination niche shift. *Nat. Commun.* 11:4019. doi: 10.1038/s41467-020-17875-1

Gould, K. S., and Lister, C. (2006). "Flavonoid functions in plants," in *Flavonoids. Chemistry, Biochemistry, and Applications*, eds Ø. M. Andersen and K. R. Markham (Boca Raton, FL: CRC Press), 397–441.

Han, M. L., Yin, J., Zhao, Y. H., Sun, X. W., Meng, J. X., Zhou, J., et al. (2020). How the color fades from *Malus halliana* flowers: Transcriptome sequencing and DNA methylation analysis. *Front. Plant Sci.* 11:576054. doi: 10.3389/fpls.2020.576054

Herman, J. J., and Sultan, S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. *Proc. Biol. Sci.* 283:20160988. doi: 10.1098/rspb.2016.0988

Herrera, C. M., and Bazaga, P. (2013). Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees. *Bot. J. Linn. Soc.* 171, 441–452. doi: 10.1111/boj.12007

Herrera, C. M., Medrano, M., and Bazaga, P. (2014). Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus*. *Mol. Ecol.* 23, 1085–1095. doi: 10.1111/mec.12679

Herrera, C. M., Pozo, M. I., and Bazaga, P. (2012). Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Mol. Ecol.* 21, 2602–2616. doi: 10.1111/j.1365-294X.2011.05402.x

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., and Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J. Exp. Bot.* 62, 2465–2483. doi: 10.1093/jxb/erq442

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am. Nat.* 165, 225–237. doi: 10.1086/426714

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Q. Rev. Biol.* 84, 131–176. doi: 10.1086/598822

Jacobsen, D. J., and Raguso, R. A. (2018). Lingering effects of herbivory and plant defenses on pollinators. *Curr. Biol.* 28, R1164–R1169. doi: 10.1016/j.cub.2018.08.010

Johnson, M. T., Campbell, S. A., and Barrett, S. C. (2015). Evolutionary interactions between plant reproduction and defense against herbivores. *Ann. Rev. Ecol. Evol. Syst.* 46, 191–213.

Kessler, A., and Chautá, A. (2020). The ecological consequences of herbivore-induced plant responses on plant–pollinator interactions. *Emerg. Top. Life Sci.* 4, 33–43. doi: 10.1042/ETLS20190121

Koski, M. H., and Galloway, L. F. (2020). Geographic variation in floral color and reflectance correlates with temperature and colonization history. *Front. Plant Sci.* 11:991. doi: 10.3389/fpls.2020.00991

Laitinen, R. A. E., and Nikoloski, Z. (2019). Genetic basis of plasticity in plants. *J. Exp. Bot.* 70, 795–804. doi: 10.1093/jxb/ery404

Lehto, W. R., and Tinghitella, R. M. (2020). Predator-induced maternal and paternal effects independently alter sexual selection. *Evolution* 74, 404–418. doi: 10.1111/evo.13906

Lev-Yadun, S., and Gould, K. S. (2008). "Role of anthocyanins in plant defence," in *Anthocyanins*, eds C. Winefield, K. Davies and K. Gould (New York, NY: Springer), 22–28. doi: 10.1007/978-0-387-77335-3_2

Losada, M., Veiga, T., Guitián, J., Guitián, J., Guitián, P., and Sobral, M. (2015). Is there a hybridization barrier between *Gentiana lutea* color morphs? *PeerJ* 3:e1308. doi: 10.7717/peerj.1308

McCall, A. C., Case, S., Espy, K., Adams, G., and Murphy, S. J. (2018). Leaf herbivory induces resistance against florivores in *Raphanus sativus*. *Botany* 96, 337–343. doi: 10.1139/cjb-2017-0153

Morita, Y., and Hoshino, A. (2018). Recent advances in flower color variation and patterning of Japanese morning glory and petunia. *Breed. Sci.* 68, 128–138. doi: 10.1270/jsbbs.17107

Narbona, E., Wang, H., Ortiz, P. L., Arista, M., and Imbert, E. (2018). Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* 20, 8–20. doi: 10.1111/plb.12575

Neylan, I. P., Dirzo, R., and Sobral, M. (2018). Cumulative effects of transgenerational induction on plant palatability to generalist and specialist herbivores. *Web Ecol.* 18, 41–46. doi: 10.5194/we-18-41-2018

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Core Team.

Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Roberts, W. R., and Roalson, E. H. (2017). Comparative transcriptome analyses of flower development in four species of Achimenes (Gesneriaceae). *BMC Genomics* 18:240. doi: 10.1186/s12864-017-3623-8

Rusman, Q., Lucas-Barbosa, D., Poelman, E. H., and Dicke, M. (2019a). Ecology of plastic flowers. *Trends Plant Sci.* 24, 725–740. doi: 10.1016/j.tplants.2019.04.007

Rusman, Q., Poelman, E. H., Nowrin, F., Polder, G., and Lucas-Barbosa, D. (2019b). Floral plasticity: herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator visitation. *Plant Cell Environ.* 42, 1882–1896. doi: 10.1111/pce.13520

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Sobel, J. M., Chen, G. F., Watt, L. R., and Schemske, D. W. (2010). The biology of speciation. *Evolution* 64, 295–315. doi: 10.1111/j.1558-5646.2009.00877.x

Sobel, J. M., and Streisfeld, M. A. (2013). Flower color as a model system for studies of plant evo-devo. *Front. Plant Sci.* 4:321. doi: 10.3389/fpls.2013.00321

Sobral, M., Veiga, T., Domínguez, P., Guitián, J. A., Guitián, P., and Guitián, J. M. (2015). Selective pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS One* 10:e0132522. doi: 10.1371/journal.pone.0132522

Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3898–3903. doi: 10.1073/pnas.071053098

Stanton, M. L. (1987). Reproductive biology of petal color variants in wild populations of *Raphanus savitus*: I. Pollinator response to color morphs. *Am. J. Bot.* 74, 178–187. doi: 10.1002/j.1537-2197.1987.tb08595.x

Strauss, S. Y., Irwin, R. E., and Lambrix, V. M. (2004). Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *J. Ecol.* 92, 132–141. doi: 10.1111/j.1365-2745.2004.00843.x

Strauss, S. Y., and Whittall, J. B. (2006). Non-pollinator agents of selection on floral traits. *Ecol. Evol. Flowers* 208, 120–138.

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Van Der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

Veiga, T., Guitián, J., Guitián, P., Guitián, J., and Sobral, M. (2015). Are pollinators and seed predators selective agents on flower color in Gentiana lutea? *Evol. Ecol.* 29, 451–464. doi: 10.1007/s10682-014-9751-6

Vekemans, X., Beauwens, T., Lemaire, M., and Roldán-Ruiz, I. (2002). Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Mol. Ecol.* 11, 139–151. doi: 10.1046/j.0962-1083.2001.01415.x

Verhoeven, K. J. F., Jansen, J. J., van Dijk, P. J., and Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol.* 185, 1108–1118. doi: 10.1111/j.1469-8137.2009.03121.x

Verhoeven, K. J. F., vonHoldt, B. M., and Sork, V. L. (2016). Epigenetics in ecology and evolution: what we know and what we need to know. *Mol. Ecol.* 25, 1631–1638. doi: 10.1111/mec.13617

Wang, L., Ran, L., Hou, Y., Tian, Q., Li, C., Liu, R., et al. (2017). The transcription factor MYB115 contributes to the regulation of proanthocyanidin biosynthesis and enhances fungal resistance in poplar. *New Phytol.* 215, 351–367. doi: 10.1111/nph.14569

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Wester, P., and Lunau, K. (2017). Plant–pollinator communication. *Adv. Bot. Res.* 82, 225–257. doi: 10.1016/bs.abr.2016.10.004

Zhang, Q., Zhao, X., and Qiu, H. (2013). "Flavones and flavonols: Phytochemistry and biochemistry," in *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*, eds K. Ramawat and J. M. Mérillon (Berlin: Springer), 1821–1847. doi: 10.1007/978-3-642-22144-6_60

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Abiotic Environment Predicts Micro- but Not Macroevolutionary Patterns of Flower Color in Monkeyflowers (Phrymaceae)

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Anthocyanin pigments are responsible for many of the vivid pink, purple, red, and blue flower colors across angiosperms and frequently vary within and between closely related species. While anthocyanins are well known to influence pollinator attraction, they are also associated with tolerance to abiotic stressors such as extreme temperatures, reduced precipitation, and ultraviolet radiation. Using a comparative approach, we tested whether abiotic variables predict floral anthocyanin in monkeyflowers (Phrymaceae) across western North America. Within two polymorphic species, we found that abiotic variables predicted flower color across their geographic ranges. In *Erythranthe discolor*, the frequency of pink flowered (anthocyanin producing) individuals was greater in populations with reduced precipitation. In *Diplacus mephiticus*, the frequency of pink flowered individuals was greater at higher elevations that had reduced precipitation and lower temperatures but less ultraviolet radiation. At the macroevolutionary scale, across two parallel radiations of North American monkeyflowers, species with floral anthocyanins (pink, purple, or red corollas) occupied areas with reduced precipitation in *Erythranthe* but not *Diplacus*. However, after accounting for phylogenetic relatedness, we found no evidence for the joint evolution of flower color and environmental affinity in either clade. We conclude that although abiotic stressors may play a role in the evolution of flower color within polymorphic species, we found no evidence that these processes lead to macroevolutionary patterns across monkeyflowers.

Keywords: anthocyanin, balancing selection, *Diplacus*, drought, *Erythranthe*, floral pigment, *Mimulus*, precipitation

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INTRODUCTION

Anthocyanin pigments cause the vivid red, blue, pink and purple colors in flowers, and are one of the most common flower color polymorphisms across angiosperms (Richards, 1986; Warren and Mackenzie, 2001). Floral anthocyanins are widely known for their role in pollinator attraction, reproductive isolation and speciation (e.g., Schemske and Bradshaw, 1999; Zufall and Rausher, 2004; Streisfield and Kohn, 2006; Hoballah et al., 2007). However, many recent studies have found that they also confer tolerance to abiotic stressors such as drought, heat, cold, and ultraviolet

radiation, with strong abiotic selection leading to trait-environment correlations (e.g., Warren and Mackenzie, 2001; Coberly and Rausher, 2003; Schemske and Bierzychudek, 2007; Dick et al., 2011; also reviewed in Strauss and Whittall, 2006). This raises the question: are abiotic stressors common drivers of flower color evolution and, if so, does this lead to macroevolutionary patterns such as the association between floral anthocyanin and abiotic stressors across species?

Anthocyanins are the last product in the flavonoid biochemical pathway and likely have an ancient origin—they are present in most land plants including mosses, ferns, gymnosperms, and angiosperms (Campanella et al., 2014). In angiosperms anthocyanins occur in both floral and vegetative tissues, and gene expression is sometimes correlated across tissue types (e.g., Albert et al., 2011; Yuan et al., 2014). Both direct and indirect selection on floral anthocyanins is therefore possible. For example, floral anthocyanins may be directly selected on due to protection of flowers or flower buds from abiotic stressors (e.g., Dick et al., 2011; Koski and Galloway, 2018; Peach et al., 2020) or indirectly through selection in vegetative tissue where anthocyanins protect plants from a variety of biotic and abiotic stressors (Warren and Mackenzie, 2001; Coberly and Rausher, 2003).

While a wide variety of environmental stressors are hypothesized to favor pigmented individuals, three in particular have been the focus of most case studies: drought, thermal extremes and UV radiation. Drought stress occurs when low soil moisture impairs normal plant growth, water relations and water use efficiency (Farooq et al., 2012). Anthocyanin accumulation in plant tissues is known to ameliorate these effects, which means that under low-water conditions, pigmented individuals may have higher fitness than unpigmented individuals (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001; Tang and Huang, 2010). For example, in a polymorphic desert annual, *Linanthus parryae*, water use efficiency was greater in individuals with anthocyanin pigmentation (blue flowers) than in those without (white flowers) (Schemske and Bierzychudek, 2001). Fitness estimates across 11 years showed that selection on flower color was strongly correlated with spring precipitation rather than pollinators.

Temperature extremes may also exert selection on floral anthocyanins. For example, in the common morning glory, *Ipomoea purpurea,* high temperatures led to a decrease in male fertility that was greater in individuals without pigmentation than in those with pigmentation (Coberly and Rausher, 2003). Low temperatures (e.g., at or below freezing) may also favor anthocyanin pigmentation in flowers. For example, populations of arctic mustard, *Parrya nudicaulis*, have a higher frequency of purple flowered individuals in locations with lower summer temperatures (Dick et al., 2011). Because floral and vegetative anthocyanin production are not correlated in this species, this could be an example of direct selection on floral pigments due to cold thermal stress. Dark floral pigments have been shown to increase the temperature of a flower by more than 5 degrees C in some species, which could impact pollinator visitation and reproduction (reviewed in van der Kooi et al., 2019).

Finally, high ultraviolet radiation, specifically UV-B, can be detrimental to developing vegetative and floral tissue (Strid et al., 1994; Koski and Ashman, 2015). Anthocyanin pigments may protect tissues from UV-B induced DNA damage by acting as a sort of "sunscreen" (Steyn et al., 2002; Mori et al., 2005). This suggests that anthocyanin pigments should be more common in regions with high UV radiation during the growing season. For example, in *Clarkia unguiculata,* floral anthocyanin concentration was greatest in areas with high UV radiation across its geographic range (Peach et al., 2020).

All of the above examples involve *within*-species comparisons, where both anthocyanin and anthocyanin-less morphs (or continuous variation in anthocyanin concentration in the case of *Clarkia unguiculata*) were present within a single population or species. It is unknown whether these micro-evolutionary processes lead to macroevolutionary patterns across species (but see Dalrymple et al., 2020). Here, we address this question using monkeyflowers (Phrymaceae), a diverse lineage of flowering plants in western North America with incredible variation in floral anthocyanins (Beardsley et al., 2004; Cooley and Willis, 2009; Streisfeld and Rausher, 2009; Yuan et al., 2014) and a wide range of environmental tolerances (Sheth and Angert, 2014). We begin by focusing within two polymorphic species (**Figure 1**), asking whether the frequency of individuals with pink flowers (caused by anthocyanins) is greater in areas with more drought, thermal stress, or UVB-radiation. We then use a comparative phylogenetic approach to test for correlated evolution of anthocyanin in primary petal tissue and abiotic affinity across 66 species in two major monkeyflower clades, *Erythranthe* Spach. and *Diplacus* Nutt.

MATERIALS AND METHODS

Study System

The family *Phrymaceae* contains over 200 described species of small annuals, perennial herbs, and woody shrubs (Barker et al., 2012; Nesom and Fraga, 2019; Liu et al., 2020). Although it has a worldwide distribution, about 75% of species are restricted to western North America and inhabit a wide variety of environments from deserts in southern California to temperate rainforests in the Pacific Northwest. The two largest genera are *Erythranthe* and *Diplacus* (N = 122 and 46 species respectively). Both genera have a center of diversity in the California floristic province and crown ages between 10 and 20 MY (data from Briscoe Runquist et al., 2016). Thus, *Erythranthe* and *Diplacus* represent parallel radiations that occurred during a period of climate change toward hotter drier summers and cooler wetter winters, i.e., during the onset of a Mediterranean climate. Across both clades, present day species occupy a wide range of climates (Grossenbacher et al., 2014; Sheth et al., 2014). While climate affinity is predicted by phylogenetic relatedness across monkeyflowers, there is substantial variation even between closest relatives (Grossenbacher et al., 2014).

Across *Erythranthe* and *Diplacus*, primary flower colors range from pink, purple, yellow, and red to white; many species also have distinctive secondary patterning or nectar



FIGURE 1 | Two focal species that are polymorphic for floral pigments: *Erythranthe discolor* and *Diplacus mephiticus*. Pink-flowered morphs have anthocyanin pigments expressed throughout the corolla tissue, whereas expression is lacking in yellow-flowered morphs.

guides. In the present study, we focused solely on primary flower color, rather than secondary patterning. In both clades, anthocyanin pigments are responsible for pink and purple corollas, carotenoid pigments for yellow, and a combination of both anthocyanins and carotenoids for red (Vickery and Olson, 1956; Hiesey et al., 1971; Streisfield and Kohn, 2006; Cooley and Willis, 2009; Yuan et al., 2014). While most flower color variation occurs between species, at least eight species are polymorphic for anthocyanin/carotenoid pigmentation with populations containing both pink (anthocyanin) and yellow (carotenoid) flowered morphs (**Figure 1** and **Supplementary Figure 1**).

Flower color has been shown to impact pollinator visitation and pollinator-mediated selection across a number of taxa (e.g., *E. lewisii* and *E. cardinalis*, Schemske and Bradshaw, 1999; *D. aurantiacus,* Streisfield and Kohn, 2006; *E. bicolor* Grossenbacher and Stanton, 2014). Overall, red flowered species are primarily visited by hummingbirds (Schemske and Bradshaw, 1999; Beardsley et al., 2003). Pink, purple and yellow flowered species are primarily visited by bees (Schemske and Bradshaw, 1999; Cooley et al., 2008; Grossenbacher and Stanton, 2014; Kelsey Byers, unpublished data). Moths, butterflies and flies are also important visitors in some taxa (e.g., Streisfield and Kohn, 2006). We know of no studies to date that assess the role of abiotic drivers of selection on flower color in either genus.

Within Species Comparisons

Population Sampling

We focused on two polymorphic annuals that represent the two major lineages in Phrymaceae: *Erythranthe discolor* and *Diplacus mephiticus* (**Figure 1**). *Erythranthe discolor* is an extremely narrow ranged endemic species in the southern Sierra Nevada, while *D. mephiticus* is widespread across much of the Sierra Nevada and extends into the Great Basin in Nevada. For both species, we defined populations as separate if they were at least 0.5 km apart. In cases where pairs of sites were sampled less than 0.5 km apart (N = 2 for *E. discolor* and N = 6 for *D. mephiticus*), we randomly removed one member of each pair from all downstream analyses.

In 2017, we conducted field surveys of *E. discolor* populations across a longitudinal gradient that transects the geographic region where both flower color morphs are present. All previously known populations in this region were sampled, as were 3 new populations that were encountered during the 2017 field surveys. At each population, we first identified the spatial extent of all non-flowering and flowering individuals, and then used a 1 by 1 meter grid system to systematically count every flowering individual within the population and recorded whether the primary corolla color was pink (anthocyanin) or yellow (no anthocyanin). The latitude and longitude was recorded from the approximate center of each population. All sampling occurred during the approximate window of peak flowering between June and early July.

To assess flower color in *Diplacus mephiticus,* we utilized herbarium specimens (N = 164). We chose to use specimens rather than field surveys mainly due to logistical reasons and to reduce the carbon footprint of our study: *D. mephiticus* populations span hundreds of kilometers and many require multiple day hiking trips. Flower color on each specimen was categorized as either yellow, pink, or polymorphic. Although flower color fades on older specimens, it is usually possible to distinguish pink from yellow. We know this because in many cases corolla color was described on the labels, allowing us to assess our accuracy using a subset of specimens (data not presented). We excluded all specimens where flower color was unclear. Populations that are polymorphic for flower color were often mounted onto separate herbarium sheets (e.g., two sheets with an a or b suffix on the collector number, or in consecutive number series), therefore records were combined and scored as polymorphic if the collector, location and date were identical. Due to the likelihood of undersampling populations that are skewed for a particular color morph, polymorphic populations may have been underscored. The latitude and longitude of each specimen was recorded either directly from the specimen, or by using detailed location information provided on the specimen label to determine the latitude and longitude on a map.

Characterizing Environmental Conditions at Each Population

We examined six environmental attributes relating to drought, temperature and UV stressors that vary across our study populations: mean annual temperature, mean annual

precipitation, precipitation during peak flower, climatic water deficit, UV-B radiation and elevation. Mean annual temperature and precipitation were extracted from the PRISM dataset using 30 year averages from 1981-2020 (PRISM Climate Group, Oregon State University, http://prism.oregonstate.edu, downloaded June 2020). The PRISM climate interpolation method performs particularly well in mountainous regions where many monkeyflowers occur (Daly et al., 2008). Climatic water deficit (CWD) values were extracted from the TerraClimate gridded data set using 30 year averages from 1981-2020 (Abatzoglou et al., 2018). CWD is a measure of how much energy availability (temperature) exceeds water supply (precipitation), with higher values indicating hotter, drier conditions, and lower values indicating cooler, wetter conditions. UV-B radiation values were extracted from gIUV dataset and represent the sum of monthly mean UVB during the highest quarter (Beckmann et al., 2014). Finally, since temperature, moisture and solar radiation likely vary along elevation gradients, elevation values were extracted from the USGS elevation point query service[1].

Statistical Analysis

To test whether the frequency of pink flowered (anthocyanin present) morphs are predicted by environmental traits across 14 *Erythranthe discolor* populations, we used beta regression models (betareg function in R package betareg, Cribari-Neto and Zeileis, 2009). Beta regression provides a flexible model for continuous response variables defined on the interval (0,1) that display both heteroscedasticity and skewness, e.g., proportional data with many values close to zero. The response variable (frequency of anthocyanin present morphs, y) was transformed prior to analysis, using a standard transformation $y(n-1)+0.5/n$ where n is the sample size (Smithson and Verkuilen, 2006) because in some cases frequency assumed values of 0 and 1. The six continuous environmental variables were treated as predictors in separate models. Because the response to environmental stressors may be non-linear, we used likelihood ratio tests to compare models with versus without a second order quadratic term and present whichever model was a better fit to our data. Models were fit using maximum likelihood with a bias correction and partial Wald tests were used to determine whether predictors were significant. We accounted for multiple comparisons ($N = 6$), using Benjamini and Hochberg (1995) correction.

To test whether *D. mephiticus* populations that differ in flower color (pink, yellow, or polymorphic) occupy distinct abiotic environments, we used analysis of variance (ANOVA, aov function in base R) or, in cases where model assumptions were not met, the non-parametric Kruskal-Wallis test (kruskal.test function in base R). We again accounted for multiple comparisons ($N = 6$), using Benjamini and Hochberg (1995) correction. In cases where the overall model was significant, we then used a *post hoc* Tukey's test or the non-parametric Dunn Test to determine which of the three population types occupied significantly distinct abiotic environments (Tukey's HSD function in base R or dunnTest function in R package FSA, Ogle et al., 2020).

[1]https://ned.usgs.gov/epqs/

Between-Species Comparisons

Phylogenetic Relationships

We utilized a previously published family level phylogeny (Grossenbacher et al., 2014) which simultaneously estimated the phylogenetic relationships and relative divergence times among *Phrymaceae* species in a Bayesian framework using the nuclear ribosomal ITS and ETS regions and chloroplast *trn*L-F region of Beardsley et al. (2004). All taxonomy was updated to current standards (Nesom and Fraga, 2019) and a sample of 900 trees from the posterior distribution was used for all downstream analyses. Trees were pruned to include only those species for which we obtained flower color and environmental data (see below), resulting in a final dataset of 27 *Erythranthe* species and 29 *Diplacus* species. We note that the topologies of these trees agreed with a recent reconstruction of the *D. aurantiacus* subclade (Stankowski et al., 2019).

Characterizing Anthocyanin Presence

To infer anthocyanin presence across species we used flower color descriptions from monographs (Grant, 1924; Thompson, 2005), taxonomic treatments (Nesom and Fraga, 2019), and herbarium specimens. We further corroborated this by visually examining and photographing corollas at multiple populations for all study species (NF and DG made these observations during field work across western North America from 2005-2020). Each species was categorized based on the predominant color of the corolla; nectar guides were excluded. For example, although *E. guttata* often has red spots on the lower corolla lobe, the predominant color is yellow so this was assigned as 'anthocyanins absent'. This resulted in three potential character states: anthocyanin (red, pink, purple flowers), no anthocyanin (yellow, orange, white flowers), or polymorphic. In cases where the upper and lower corolla lobes differed in predominant color, we assigned the species as 'anthocyanin' if either the upper or lower corolla lobes were red, pink or purple (e.g., *E. shevockii*).

Characterizing Environmental Affinity

To identify the average environmental conditions occupied by each species, we utilized a previously curated data set of known occurrences based on herbarium records (**Figure 2**; Grossenbacher et al., 2014). The average number of occurrences per species was 100 (+ -SE = 30.0, maximum = 1760, minimum = 6). In cases where within-species' occurrences were within 1 km of one another, occurrences were deleted at random to restrict observations to one record per environmental grid cell in order to reduce sampling bias. We then extracted environmental values for all remaining occurrences and calculated the mean environmental values for each species across all six environmental traits described above.

Statistical Analyses

To test whether species that differ in floral anthocyanins occupy distinct abiotic environments, we used ANOVA or, in cases where model assumptions were not met, the non-parametric Kruskal-Wallis test. Species' floral anthocyanin (anthocyanin, non-anthocyanin, or polymorphic) was treated as a fixed factor and the six environmental traits were treated as response

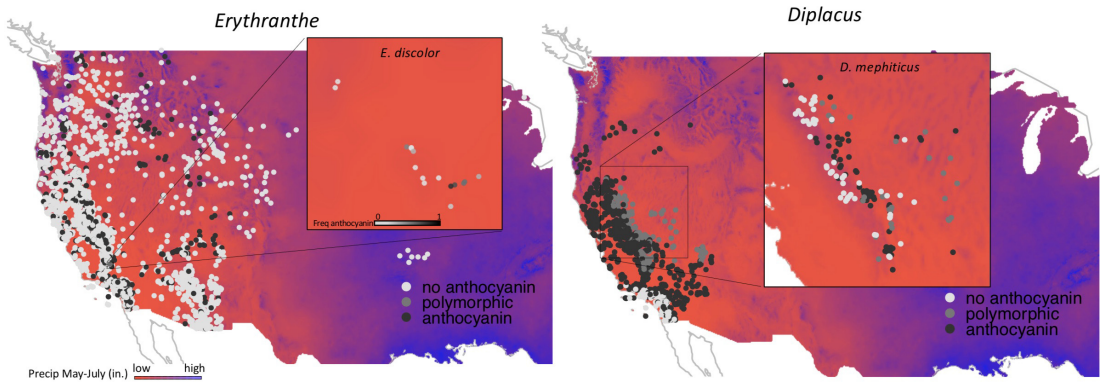


FIGURE 2 | Occurrence data for *Erythranthe* **(left panel)** and *Diplacus* **(right panel)** with filled circle color representing anthocyanin pigmentation (see legend). Background colors represent precipitation during peak flowering with red indicating regions with the lowest precipitation and blue indicating the highest. Insets represent population level data for the two focal species that are polymorphic for flower color.

variables in six separate models. We accounted for multiple comparisons (N = 6), using Benjamini and Hochberg (1995) correction. In cases where the overall model was significant, we used a *post hoc* Tukey's test or the non-parametric Dunn Test to determine which of the three anthocyanin categories significantly differed from one another. The same functions and R packages were used here as for the within species analyses described above.

To explicitly test whether there is evidence of correlated evolution of corolla anthocyanins and environmental traits, we used a phylogenetic ANOVA (function aov.phylo, R package geiger, Pennell et al., 2014). Phylogenetic uncertainty was taken into account by performing these tests on a sample of trees from the posterior distribution (N = 900). Average P-values are reported.

Finally, we tested whether relatedness within *Erythranthe* and *Diplacus* predicts floral anthocyanins, i.e., whether there is phylogenetic signal of flower color. Treating floral anthocyanins as a discrete trait with three character states, we used Maddison and Slatkin (1991) to determine whether the observed number of character state transitions was significantly less than when species names were randomized across the phylogeny (N = 999 randomization; R code from Bush et al., 2016). Again, phylogenetic uncertainty was taken into account by performing this test on a sample of trees from the posterior distribution (N = 900). Average observed transitions and P-values are reported.

All statistical analyses described above were performed in R version 4.0.2 (R Core Team, 2020).

RESULTS

Across 14 populations of *Erythranthe discolor* (**Figure 2** inset), the frequency of pink flowered individuals (anthocyanin present) was significantly greater in areas with less annual precipitation and less precipitation during peak flowering May-July (**Figure 3** and **Table 1**). None of the four other environmental variables were significant predictors of flower color after accounting for multiple test comparisons (**Table 1**).

Across 158 populations of *Diplacus mephiticus* (**Figure 2** inset), flower color was significantly predicted by all six environmental variables (**Figure 3** and **Table 1**). Pink flowered populations occurred in cooler areas with significantly less precipitation than yellow flowered populations, and occurred in areas with less UV radiation and at higher elevations than either yellow or polymorphic populations. Polymorphic populations on the other hand occurred in areas that are both warmer than pink populations, with less precipitation than yellow populations, and had higher climatic water deficit than either pink or yellow populations.

At the species level across the two largest clades of monkeyflowers in western North America (**Figures 2**, **4**), we found that pink flowered *Erythranthe* species occupied areas with significantly less precipitation during peak flower (May-July) than yellow flowered *Erythranthe*, while none of the other traits were significant for either *Erythranthe* or *Diplacus* (**Table 1** and **Figure 5**). We found no evidence of correlated evolution of flower color and any climate traits (phylogenetic ANOVA $P > 0.05$, **Table 1**).

Finally, we found that floral anthocyanins displayed significant phylogenetic signal in *Erythranthe* but not *Diplacus*. In both clades, there were on average 6 observed transitions in flower color across the posterior distribution of trees—in *Erythranthe*, this was significantly less than when species were randomized across the phylogeny ($P < 0.001$), but not in *Diplacus* ($P = 0.201$).

DISCUSSION

Our results are consistent with abiotic selection on flower color within populations — flower color in two polymorphic monkeyflowers was correlated with abiotic environmental conditions across the landscape. However, we found no evidence that microevolutionary processes lead to clade-wide macroevolutionary patterns—the evolution of flower color and climate affinity were uncorrelated across monkeyflowers in western North America.

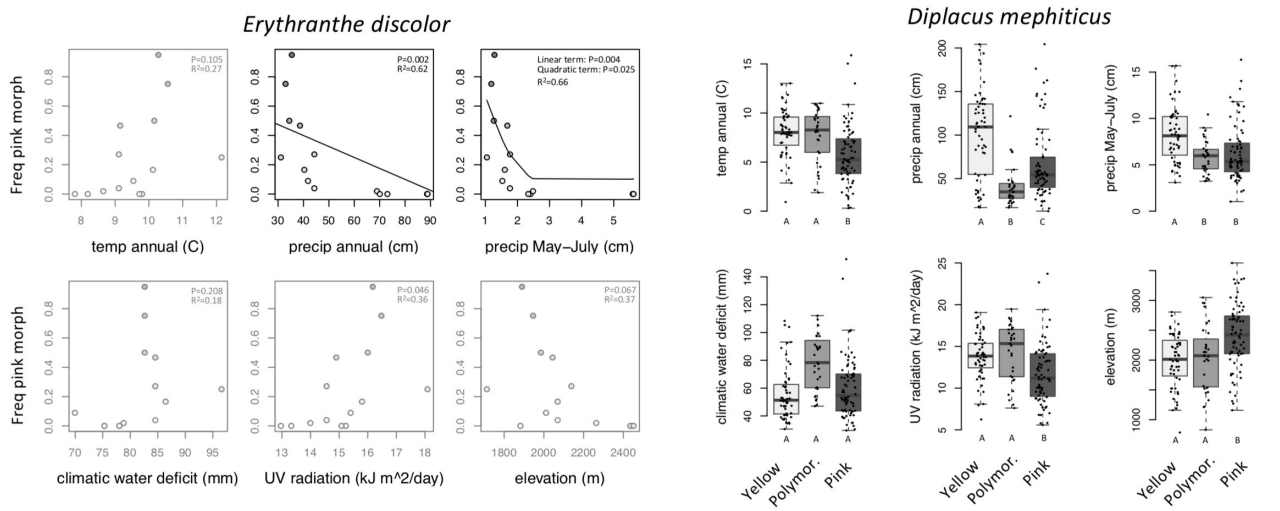


FIGURE 3 | Within-species variation in climate, UV, and elevation affinity by primary flower color category, displayed as boxplots. Filled circles represent observed data for each color category. Lines represent the predicted slope from beta regression and letters indicate *post hoc* comparisons where $P < 0.05$. Opaque plots are those without significant predictors after correcting for multiple tests. See **Table 1** for statistical results.

TABLE 1 | Results of models analyzing the relationship between abiotic environmental variables and flower color.

	Within species	
Predictor variable:	***Erythranthe discolor* (*N* = 14 populations)**	***Diplacus mephiticus* (*N* = 158 populations)**
Temp annual	Beta Reg, $Z = 1.619$, $P = 0.105$	Kruskal, $X^2 = 25.70$, $P <$ **0.001**
Precip annual	Beta Reg, $Z = -3.239$, P = **0.002**	Kruskal, $X^2 = 34.39$, $P <$ **0.001**
Precip May–July	Beta Reg, linear: $Z = 2.846$, $P =$ **0.004**; quadratic: $Z = -2.58$, $P = 0.025$	Kruskal, $X^2 = 20.41$, $P <$ **0.001**
Climatic water deficit	Beta Reg, $Z = 1.259$, $P = 0.208$	Kruskal, $X^2 = 24.53$, $P <$ **0.001**
UV radiation	Beta Reg, $Z = 2.855$, $P = 0.046$	Kruskal, $X^2 = 20.21$, $P <$ **0.001**
Elevation	Beta Reg, $Z = -1.832$, $P = 0.067$	ANOVA, $F = 11.32$, $P <$ **0.001**
	Between species	
Predictor variable:	***Erythranthe* (*N* = 37 species)**	***Diplacus* (*N* = 29 species)**
Temp annual	ANOVA, $F = 0.53$, $P = 0.470$; phy.anova, $P = 0.836$	ANOVA, $F = 3.67$, $P = 0.040$; phy.anova, $P = 0.105$
Precip annual	ANOVA, $F = 1.02$, $P = 0.319$; phy.anova, $P = 0.769$	ANOVA, $F = 1.16$, $P = 0.328$; phy.anova, $P = 0.643$
Precip May–July	Kruskal, $X^2 = 6.32$, $P =$ **0.004**; phy.anova, $P = 0.445$	Kruskal, $X^2 = 5.70$, $P = 0.058$; phy.anova, $P = 0.191$
Climatic water deficit	ANOVA, $F = 5.68$, $P = 0.023$; phy.anova, $P = 0.473$	Kruskal, $X^2 = 2.52$, $P = 0.284$; phy.anova, $P = 0.723$
UV radiation	ANOVA, $F = 0.47$, $P = 0.499$; phy.anova, $P = 0.845$	ANOVA, $F = 1.23$, $P = 0.309$; phy.anova, $P = 0.460$
Elevation	ANOVA, $F = 2.48$, $P = 0.125$; phy.anova, $P = 0.645$	ANOVA, $F = 4.51$, $P = 0.021$; phy.anova, $P = 0.068$

Note that for phylogenetic ANOVA, we report the mean P-value across 900 Phrymaceae phylogenies sampled from the Bayesian posterior distribution of trees. Bold P-values are significant ($P < 0.05$) after Benjamini and Hochberg correction for multiple test comparisons.

Within two polymorphic monkeyflowers, the frequencies of pink flowers were greater in areas with less precipitation. This is consistent with other polymorphic species where floral anthocyanins were favored in drier environments (e.g., *Linanthus parryae*, Schemske and Bierzychudek, 2001; *Cirsium palustre*, *Digitalis purpurea*, *Holcus lanatus*, *Polygonum persicaria*, and *Vicia sepium,* Warren and Mackenzie, 2001; *Butomus umbellatus,* Tang and Huang, 2010). Together this suggests that across diverse lineages, floral anthocyanins can either directly or indirectly lead to drought tolerance. In heterogeneous landscapes the benefit of anthocyanins may be especially great, and polymorphisms are predicted to be maintained by balancing selection (Warren and Mackenzie, 2001). This may be especially true in the American west, where interannual variation in rainfall is common. While our study examined only two polymorphic monkeyflower species, there are at least six additional species with population-level variation in floral anthocyanins: *E. rubella*, *E. barbata*, *D. leptaleus, D. bicolor*, and *D. parryi*. Future studies using a mix of comparative, experimental and genomic data are needed to uncover the extent of the relationship between anthocyanins, drought stress and balancing selection.

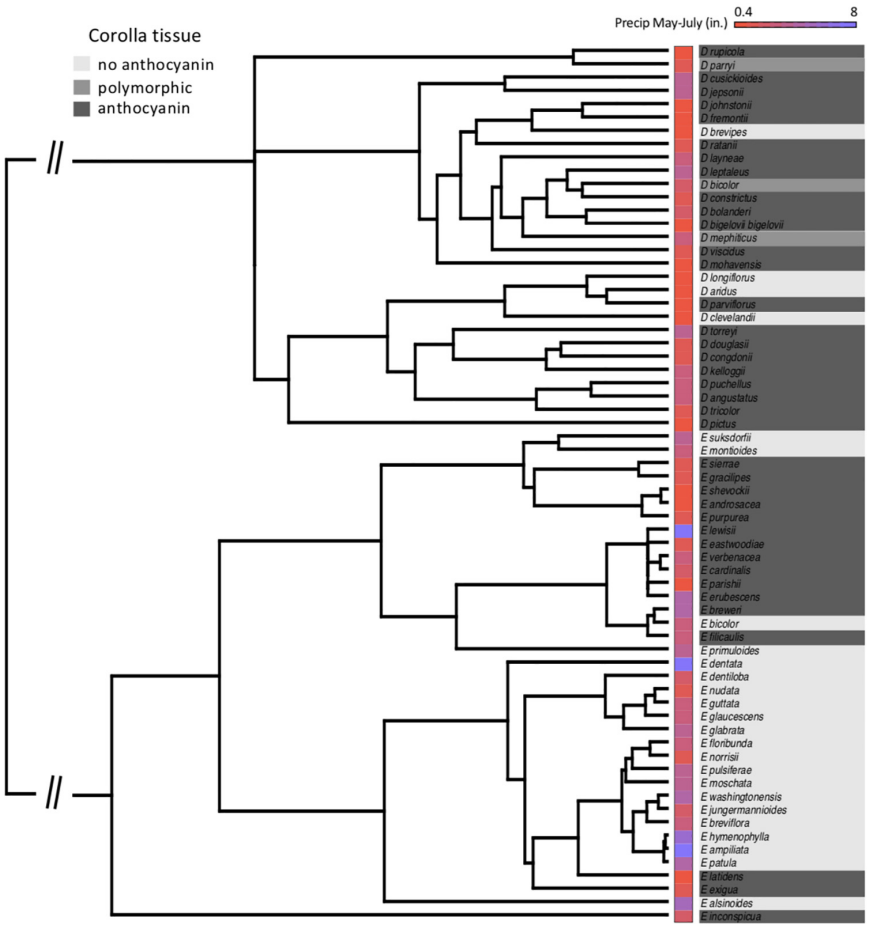


FIGURE 4 | Species' relationships in clades *Diplacus* and *Erythranthe*, with floral anthocyanin and average precipitation during peak flowering (May–July) indicated. Note that the phylogeny is pruned to only include species in the present study ($N = 66$).

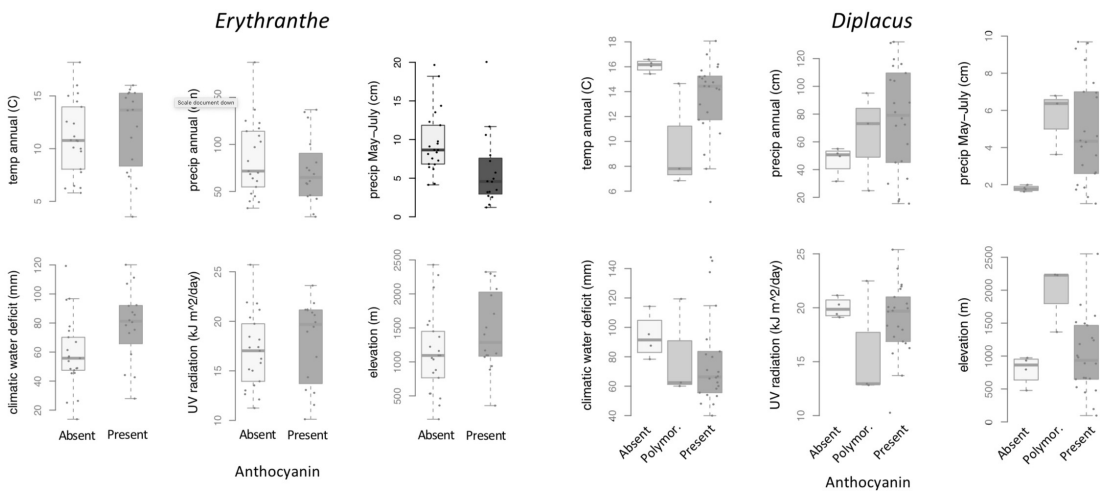


FIGURE 5 | Between-species variation in climate, UV, and elevation affinity by anthocyanin category, displayed as boxplots. Filled circles represent observed data for each anthocyanin category. Opaque plots are those without significant predictors after correcting for multiple tests. See **Table 1** for statistical results.

Temperature extremes are another factor that may favor floral anthocyanins. While some previous studies have found that floral anthocyanins protect against high temperatures (Coberly and Rausher, 2003), we found the opposite in *D. mephiticus*—the frequency of pink flowers was greater in areas with *lower* annual temperatures. There is, however, evidence that anthocyanins may also protect plants in near freezing environments (McKown et al., 1996) favoring anthocyanin morphs in cold environments (e.g., Dick et al., 2011). Consistent with this, pink flowered populations of *D. mephiticus* occur at higher elevations than yellow-flowered populations (above 2000 m on average), where near freezing temperatures are more likely to occur during the growing season of this short lived annual species.

Ultraviolet radiation predicted flower color in *D. mephiticus*, but the relationship was opposite what we expected—pink-flowered populations occurred in areas with less UV radiation than yellow-flowered populations. This was surprising given the fact that these populations also occupy higher elevations where UV radiation is generally thought to be more intense but is perhaps explained by more frequent cloud cover. Other studies have also found contradictory patterns regarding UV radiation. For example, in the Neotropical genus *Ruellia*, flavone concentration (which was correlated with floral anthocyanins) was greatest in populations at high latitudes that have correspondingly less UV radiation than populations near the equator (Tripp et al., 2018). The authors concluded that this may be due to flavonoids being selected on by other bioclimatic factors associated with latitudinal gradients, perhaps more so than UV radiation. A similar phenomenon could be occurring with *D. mephiticus*. Environmental factors that are spatially correlated make it difficult to determine the specific factors that drive the evolution of floral anthocyanins. Experimental studies under controlled environmental conditions are needed to tease environmental factors apart and determine their relative importance.

In contrast with the population level patterns described above, we found no support for abiotic drivers of floral anthocyanins at the macroevolutionary scale. We can think of three potential explanations for this finding. First, despite the role of floral anthocyanins in stress tolerance, it could be that pollinator-mediated selection is the stronger agent at the macroevolutionary scale where it can lead to reproductive isolation, speciation and lineage divergence. For example, in the *Diplacus auranticus* species complex, red flowered forms containing anthocyanins and carotenoids are preferred by hummingbirds, while yellow flowered forms containing only carotenoids are preferred by hawkmoths (Streisfield and Kohn, 2006), which could contribute to reproductive isolation and speciation in this complex. Future macroevolutionary studies that jointly examine pollinators and abiotic tolerances are needed to determine their relative contributions to flower color evolution. Second, perhaps the role of anthocyanins in stress tolerance most commonly operates under balancing selection in highly heterogeneous environments, rather than as directional selection. Balancing selection is less likely to lead to changes between lineages. Finally, because flower color is a conserved trait in monkeyflowers, it could be that there is simply low power to detect correlated evolution between flower color and abiotic traits in this system. It is therefore important to explore this question in other clades, perhaps with more frequent transitions in flower color, to determine the role of abiotic selection in shaping floral anthocyanin patterns across angiosperms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors contributed to developing the study. NF and MM conducted all within-species data collection and analysis. LM and DG conducted all family-level data collection and analysis. NF, LM, and DG co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.636133/full#supplementary-material

REFERENCES

Abatzoglou, J. T., Dobrowski, S. Z., Parks, S. A., and Hegewisch, K. C. (2018). TerraClimate, a high-resolution global dataset of monthly climate and climatic water balance from 1958–2015. *Sci. Data* 5:170191.

Albert, N. W., Lewis, D. H., Zhang, H., Schwinn, K. E., Jameson, P. E., and Davies, K. M. (2011). Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. *Plant J.* 65, 771–784. doi: 10.1111/j.1365-313x.2010.04465.x

Barker, W. R., Nesom, G. L., Beardsley, P. M., and Fraga, N. S. (2012). A taxonomic conspectus of *Phrymaceae*: a narrowed circumscription for *Mimulus*, new and resurrected genera, and new names and combinations. *Phytoneuron* 39, 1–60. doi: 10.1007/978-3-319-23534-9_1

Beardsley, P. M., Schoenig, S. E., Whittall, J. B., and Olmstead, R. G. (2004). Patterns of evolution in western north american *Mimulus* (*Phrymaceae*). *Am. J. Bot.* 91, 474–489. doi: 10.3732/ajb.91.3.474

Beardsley, P. M., Yen, A., and Olmstead, R. G. (2003). AFLP phylogeny of *Mimulus* section *Erythranthe* and the evolution of hummingbird pollination. *Evolution* 57, 1397–1410. doi: 10.1111/j.0014-3820.2003.tb00347.x

Beckmann, M., Václavík, T., Manceur, A. M., Šprtová, L., von Wehrden, H., Welk, E., et al. (2014). gl UV: a global UV-B radiation data set for macroecological studies. *Methods Ecol. Evol.* 5, 372–383. doi: 10.1111/2041-210x.12168

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Briscoe Runquist, R., Grossenbacher, D., Porter, S., Kay, K., and Smith, J. (2016). Pollinator-mediated assemblage processes in California wildflowers. *J. Evol. Biol.* 29, 1045–1058. doi: 10.1111/jeb.12845

Bush, S. E., Weckstein, J. D., Gustafsson, D. R., Allen, J., DiBlasi, E., Shreve, S. M., et al. (2016). Unlocking the black box of feather louse diversity: a molecular phylogeny of the hyper-diverse genus *Brueelia*. *Mol. Phylogenet. Evol.* 94, 737–751. doi: 10.1016/j.ympev.2015.09.015

Campanella, J. J., Smalley, J. V., and Dempsey, M. E. (2014). A phylogenetic examination of the primary anthocyanin production pathway of the Plantae. *Bot. Stud.* 55, 1–10.

Coberly, L. C., and Rausher, M. D. (2003). Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Mol. Ecol.* 12, 1113–1124. doi: 10.1046/j.1365-294x.2003.01786.x

Cooley, A. M., Carvallo, G., and Willis, J. H., (2008). Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean *Mimulus*. *Ann. Bot.* 101, 641–650. doi: 10.1093/aob/mcn014

Cooley, A. M., and Willis, J. H. (2009). Genetic divergence causes parallel evolution of flower color in Chilean *Mimulus*. *New Phyt.* 183, 729–739. doi: 10.1111/j.1469-8137.2009.02858.x

Cribari-Neto, F., and Zeileis, A. (2009). *Beta Regression in R. Research Report Series/Department of Statistics and Mathematics, 98*. Vienna: WU Vienna University of Economics and Business.

Dalrymple, R. L., Kemp, D. J., Flores-Moreno, H., Laffan, S. W., White, T. E., Hemmings, F. A., et al. (2020). Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phyt.* 228, 1972–1985. doi: 10.1111/nph.16737

Daly, C., Halbleib, M., Smith, J. I., Gibson, W. P., Doggett, M. K., Taylor, G. H., et al. (2008). Physiographically sensitive mapping of climatological temperature and precipitation across the conterminous United States. *Int. J. Climatol.* 28, 2031–2064. doi: 10.1002/joc.1688

Dick, C. A., Buenrostro, J., Butler, T., Carlson, M. L., Kliebenstein, D. J., and Whittall, J. B. (2011). Arctic mustard flower color polymorphism controlled by petal-specific downregulation at the threshold of the anthocyanin biosynthetic pathway. *PLoS One* 6:e18230. doi: 10.1371/journal.pone.0018230

Farooq, M., Hussain, M., Wahid, A., and Siddique, K. H. M. (2012). "Drought stress in plants: an overview," in *Plant Responses to Drought Stress*, ed. R. Aroca (Berlin: Springer), 1–33. doi: 10.1007/978-3-642-32653-0_1

Grant, A. L. (1924). A monograph of the genus *Mimulus*. *Ann. Missouri Bot. Gard.* 11, 99–380. doi: 10.2307/2394024

Grossenbacher, D. L., and Stanton, M. L. (2014). Pollinator-mediated competition influences selection for flower-color displacement in sympatric monkeyflowers. *Am. J. Bot.* 101, 1915–1924. doi: 10.3732/ajb.1400204

Grossenbacher, D. L., Veloz, S. D., and Sexton, J. P. (2014). Niche and range size patterns suggest that speciation begins in small, ecologically diverged populations in North American monkeyflowers (*Mimulus* spp.). *Evolution* 68, 1270–1280. doi: 10.1111/evo.12355

Hiesey, W., Nobs, M., and Bjorkman, O. (1971). *Experimental Studies on the Nature of Species: 5. Biosystematics, Genetics and Physiological Ecology of the Erythranthe Section of Mimulus*. Washington, DC: Carnegie Institution.

Hoballah, M. E., Gübitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., et al. (2007). Single gene–mediated shift in pollinator attraction in *Petunia*. *Plant Cell* 19, 779–790. doi: 10.1105/tpc.106.048694

Koski, M. H., and Ashman, T. L. (2015). Floral pigmentation patterns provide an example of Gloger's rule in plants. *Nat. Plants* 1, 1–5.

Koski, M. H., and Galloway, L. F. (2018). Geographic variation in pollen color is associated with temperature stress. *New Phyt.* 218, 370–379. doi: 10.1111/nph.14961

Liu, B., Tan, Y. -H., Liu, S., Olmstead, R. G., Min, D. -Z., Chen, Z. -D., et al. (2020). Phylogenetic relationships of Cyrtandromoea and Wightia revisited: a new tribe in *Phrymaceae* and a new family in Lamiales. *J. Syst. Evol.* 58, 1–17. doi: 10.1111/jse.12513

Maddison, W. P., and Slatkin, M. (1991). Null models for the number of evolutionary steps in a character on a phylogenetic tree. *Evolution* 45, 1184–1197. doi: 10.2307/2409726

McKown, R., Kuroki, G., and Warren, G. (1996). Cold responses of Arabidopsis mutants impaired in freezing tolerance. *J. Exp. Bot.* 47, 1919–1925. doi: 10.1093/jxb/47.12.1919

Mori, M., Yoshida, K., Ishigaki, Y., Matsunaga, T., Nikaido, O., Kameda, K., et al. (2005). UV-B protective effect of a polyacylated anthocyanin, HBA, in flower petals of the blue morning glory, *Ipomoea tricolor* cv. Heavenly Blue. *Bioorg. Med. Chem.* 13, 2015–2020. doi: 10.1016/j.bmc.2005.01.011

Nesom, G. L., and Fraga, N. S. (2019). "*Erythranthe*," in *Flora of North America, North of Mexico*, Vol. 17, (New York, NY: Oxford University Press).

Ogle, D. H., Wheeler, P., and Dinno, A. (2020). *FSA: Fisheries Stock Analysis. R package version 0.8. 30.9000*.

Peach, K., Liu, J. W., and Mazer, S. J. (2020). Climate predicts UV Floral pattern size, anthocyanin concentration, and pollen performance in *Clarkia unguiculata*. *Front. Plant. Sci.* 11:847. doi: 10.3389/fpls.2020.00847

Pennell, M. W., Eastman, J. M., Slater, G. J., Brown, J. W., Uyeda, J. C., FitzJohn, R. G., et al. (2014). geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30, 2216–2218. doi: 10.1093/bioinformatics/btu181

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Richards, A. J. (1986). *Plant Breeding Systems*. London: George Allen and Unwin press.

Schemske, D., and Bierzychudek, P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisted. *Evolution* 55, 1269–1282. doi: 10.1554/0014-3820(2001)055[1269:peofci]2.0.co;2

Schemske, D. W., and Bradshaw, H. D. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci. U.S.A.* 96, 11910–11915. doi: 10.1073/pnas.96.21.11910

Schemske, D. W., and Bierzychudek, P., (2007). Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right?. *Evolution* 61, 2528–2543. doi: 10.1111/j.1558-5646.2007.00219.x

Sheth, S. N., and Angert, A. L. (2014). The evolution of environmental tolerance and range size: a comparison of geographically restricted and widespread *Mimulus*. *Evolution* 68, 2917–2931. doi: 10.1111/evo.12494

Sheth, S. N., Jiménez, I., and Angert, A. L. (2014). Identifying the paths leading to variation in geographical range size in western North American monkeyflowers. *J. Biogeogr.* 41, 2344–2356. doi: 10.1111/jbi.12378

Smithson, M., and Verkuilen, J. (2006). A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. *Psychol. Methods* 11, 54–71. doi: 10.1037/1082-989x.11.1.54

Stankowski, S., Chase, M. A., Fuiten, A. M., Rodrigues, M. F., Ralph, P. L., and Streisfeld, M. A. (2019). Widespread selection and gene flow shape the genomic landscape during a radiation of monkeyflowers. *PLoS Biol.* 17:e30000391. doi: 10.1371/journal.pbio.3000391

Steyn, W. J., Wand, S. J. E., Holcroft, D. M., and Jacobs, G. (2002). Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytol.* 155, 349–361. doi: 10.1046/j.1469-8137.2002.00482.x

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: OUP Oxford), 120–138.

Streisfeld, M. A., and Rausher, M. D. (2009). Altered trans-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. *Mol. Biol. Evol.* 26, 433–444. doi: 10.1093/molbev/msn268

Streisfeld, M. A., and Kohn, J. R. (2006). Envrionment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evol. Biol.* 20, 122–132. doi: 10.1111/j.1420-9101.2006.01216.x

Strid, Å, Chow, W. S., and Anderson, J. M. (1994). UV-B damage and protection at the molecular level in plants. *Photosynth. Res.* 39, 475–489. doi: 10.1007/bf00014600

Tang, X. X., and Huang, S. Q. (2010). Fluctuating selection by water level on gynoecium colour polymorphism in an aquatic plant. *Ann. Bot.* 106, 843–848. doi: 10.1093/aob/mcq172

Thompson, D. M. (2005). Systematics of *Mimulus* subgenus Schizoplacus. American Society of Plant Taxonomists. *Syst. Bot. Monogr.* 75, 1–213.

Tripp, E. A., Zhuang, Y., Schreiber, M., Stone, H., and Berardi, A. E. (2018). Evolutionary and ecological drivers of plant flavonoids across a large latitudinal gradient. *Mol. Phylogenet. Evol.* 128, 147–161. doi: 10.1016/j.ympev.2018.07.004

van der Kooi, C. J., Kevan, P. G., and Koski, M. H. (2019). The thermal ecology of flowers. *Ann. Bot.* 124, 343–353.

Vickery, R. K., and Olson, R. L. (1956). Flower color inheritance in the *Mimulus cardinalis* complex. *J. Hered.* 47, 195–199. doi: 10.1093/oxfordjournals.jhered.a106635

Warren, J., and Mackenzie, S. (2001). Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.* 151, 237–241. doi: 10.1046/j.1469-8137.2001.00159.x

Yuan, Y. W., Sagawa, J. M., Frost, L., Vela, J. P., and Bradshaw, H. D. (2014). Transcriptional control of floral anthocyanin pigmentation in monkeyflowers (*Mimulus*). *New Phytol.* 204, 1013–1027. doi: 10.1111/nph.12968

Zufall, R. A., and Rausher, M. D. (2004). Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* 428, 847–850. doi: 10.1038/nature02489

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Evolution of the Subgroup 6 *R2R3-MYB* Genes and Their Contribution to Floral Color in the Perianth-Bearing Piperales

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Flavonoids, carotenoids, betalains, and chlorophylls are the plant pigments responsible for floral color. Anthocyanins, a class of flavonoids, are largely responsible for the red, purple, pink, and blue colors. *R2R3-MYB* genes belonging to subgroup 6 (SG6) are the upstream regulatory factors of the anthocyanin biosynthetic pathway. The canonical members of these genes in *Arabidopsis* include *AtMYB75*, *AtMYB90*, *AtMYB113,* and *AtMYB114*. The Aristolochiaceae is an angiosperm lineage with diverse floral groundplans and perianth colors. *Saruma henryi* exhibits a biseriate perianth with green sepals and yellow petals. All other genera have sepals only, with colors ranging from green (in *Lactoris*) to a plethora of yellow to red and purple mixtures. Here, we isolated and reconstructed the SG6 *R2R3-MYB* gene lineage evolution in angiosperms with sampling emphasis in Aristolochiaceae. We found numerous species-specific duplications of this gene lineage in core eudicots and local duplications in Aristolochiaceae for *Saruma* and *Asarum*. Expression of SG6 *R2R3-MYB* genes examined in different developmental stages and plant organs of four Aristolochiaceae species, largely overlaps with red and purple pigments, suggesting a role in anthocyanin and flavonoid synthesis and accumulation. A directed RNA-seq analysis corroborated our RT-PCR analyses, by showing that these structural enzymes activate during perianth development in *Aristolochia fimbriata* and that the regulatory genes are expressed in correlation with color phenotype. Finally, the reconstruction of the flavonoid and anthocyanin metabolic pathways using predicted peptides from transcriptomic data show that all pivotal enzymes are present in the analyzed species. We conclude that the regulatory genes as well as the biosynthetic pathway are largely conserved across angiosperms. In addition, the Aristolochiaceae emerges as a remarkable group to study the genetic regulatory network for floral color, as their members exhibit an outstanding floral diversity with elaborate color patterns and the genetic complement for SG6 *R2R3-MYB* genes is simpler than in core eudicot model species.

Keywords: anthocyanins, Aristolochiaceae, *Asarum*, flavonoids, floral color, petaloid sepals, *Saruma*, subgroup 6 *R2R3-MYB* genes

INTRODUCTION

The floral color palette is remarkable across angiosperms and can be linked to pollinator attraction during anthesis playing a key role in reproduction (Frey et al., 2011). Although all floral parts accumulate pigments, color display shifts during development are striking in the perianth. The chlorophylls, the carotenoids, the betalains, and the flavonoids are the primary plant pigments synthesized. Under different environmental and developmental conditions, these can be found in both vegetative and reproductive plant organs. The chlorophylls are produced in the chloroplast, are green and are required for photosynthesis, but are not leaf exclusive and can be present in the flowers (Narbona et al., 2014). The carotenoids are lipid-soluble molecules also produced in the chloroplast, which are responsible for the yellow, red, and orange colors, and are involved in the maintenance of the photosynthetic apparatus integrity (Tanaka et al., 2008; Chatham et al., 2019). On the other hand, betalains and flavonoids are both water-soluble molecules. Betalains are only present in members of the order Caryophyllales and are responsible for red and violet to yellow and orange hues. Betalains and anthocyanins are mutually exclusive since they do not occur simultaneously in any plant (Clement et al., 1994). There are many types of flavonoids such as flavonols, flavones, anthocyanins, and proanthocyanidins (Matsui and Walker, 2019). Flavonoids, in general, play various roles including the attraction of pollinators, the quenching of UV light (Tanaka et al., 2008) and protection against stress (Winkel-Shirley, 2001, 2002), such as resistance to aluminum toxicity (Kidd et al., 2001) or stress response by stomatal opening control *via* auxins (Dietrich et al., 2001). In particular, anthocyanins are responsible for colors such as red, blue, purple, and pink in different plant structures, but especially in floral organs (Chatham et al., 2019). In flowers, anthocyanins are the primary attractants for insects, birds and other biotic vectors aiding in reproduction (Jackman and Smith, 1996; Kong et al., 2003). Similarly, anthocyanin-derived fruit color can increase frugivory and seed dispersal. Anthocyanins are glycosides of anthocyanidins (Holton and Cornish, 1995), and their colors can change according to pH, as red tones prevail in acidic conditions whereas blue tones are characteristic of alkaline conditions (Calderaro et al., 2020). The most common anthocyanidins found in anthocyanins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Clifford, 2000). Anthocyanins are made from a C6-C3-C6 skeleton from which different modifications occur. Both, the type and the color of the resulting anthocyanin depend primarily on the addition of methoxyl or hydroxyl groups to the B ring (Chatham et al., 2019).

As many other metabolic plant routes, the flavonoid biosynthetic pathway, responsible for anthocyanidin production, is regulated at the transcriptional level. A MBW (MYB-bHLH-WD) transcriptional activation complex controls the latter steps of the flavonoid pathway (Schwinn et al., 2016). This complex is the result of the physical interaction between a MYB transcription factor, a basic Helix-Loop-Helix (bHLH) factor and a WD40-repeat protein (González et al., 2008; Zhang and Schrader, 2017). Here we largely concentrate on the *MYB* genes, which are a large family of transcription factors (TFs) found in most eukaryotes (Klempnauer et al., 1982). In plants, *MYB* genes are divided into four types: *4R-MYB*, *3R-MYB*, *"MYB-related,"* and the *R2R3-MYB*. The *R2R3-MYB* clade is the largest and most functionally diverse type, as these genes control the production of primary and secondary metabolites and the specialization of epidermal cells as trichomes or root hairs, among other functions (Higginson et al., 2003; Tominaga et al., 2008). The *R2R3-MYB* TFs can act as transcriptional activators (Noda et al., 1994) or repressors (Park et al., 2008). The classification of *R2R3-MYB* genes comes primarily from *Arabidopsis thaliana* homologs (Kranz et al., 1998; Romero et al., 1998; Stracke et al., 2001). Stracke et al. (2001), designated subgroups based on 125 sequences, using function and specific motifs outside the MYB domain as main classifiers. The R2R3-MYB proteins receive their name based on the R2 and R3 domains found in the N-terminal portion of the protein. Each domain contains around 53 amino acids and forms three alpha-helices with the help of three spaced tryptophan residues that form a hydrophobic cluster (Stracke et al., 2001). *R2R3-MYB* genes, known to play roles in the early stages of the anthocyanin biosynthesis pathway, have been assigned to Subgroup 6 (hereafter referred to as SG6), which include the *Arabidopsis* paralogs *AtMYB75*, *AtMYB90*, *AtMYB113*, and *AtMYB114*.

The recruitment of *R2R3-MYB* gene homologs in pigmentation has been studied in *Antirrhinum*, *Clarkia*, *Iochroma*, *Ipomoea*, *Lilium*, *Mimulus*, *Oenanthe*, *Petunia*, *Phalaenopsis,* and various Rosaceae (Bradshaw et al., 1995; Quattrocchio et al., 1999; Zufall and Rausher, 2003; Morita et al., 2006; Schwinn et al., 2006; Lin-Wang et al., 2010; Shang et al., 2011; Lowry et al., 2012; Wu et al., 2013; Hsu et al., 2015; Pérez-Díaz et al., 2016; Yamagishi, 2016; Feng et al., 2018; Gates et al., 2018; Ding et al., 2020; Lin and Rausher, 2020). In contrast, no studies are available linking *R2R3-MYB* homologs to color production in early divergent angiosperms. The only available study by Zhang et al. (2020) in Nymphaeaceae is concentrated in the flavonoid biosynthetic pathway in *Nymphaea colorata* and *N. nucifera*. In these species, the flavonoid pathway enzymes are more expressed in blue petals compared to white petals, and very specific delphinidin molecules [delphinidin 3′-O-(2″-O-galloyl-6″-O-acetyl-B-galactopyranoside)] have been found in the blue petals of *N. colorata* (Zhang et al., 2020).

The Aristolochiaceae includes all perianth-bearing Piperales, namely, *Aristolochia, Asarum, Hydnora, Lactoris, Prosopanche, Thottea,* and *Saruma* (Wanke et al., 2006; **Figure 1**). The monotypic *Saruma* is the only genus having a biseriate perianth formed by three green sepals and three yellow petals (González and Stevenson, 2000; Pabón-Mora et al., 2020). The remaining genera, *Aristolochia*, *Asarum, Hydnora, Lactoris, Prosopanche,* and *Thottea* have three sepals partially or totally fused with each other, exhibiting different colors (Pabón-Mora et al., 2020). In *Asarum,* sepals are usually dark purple but some species exhibiting yellow sepals can be found. Sepals in *Hydnora* and *Prosopanche*, the only holoparasitic members of the family, exhibit color ranges from dark purple and bright red or orange tones to white or cream (Musselman and Visser, 1989). The

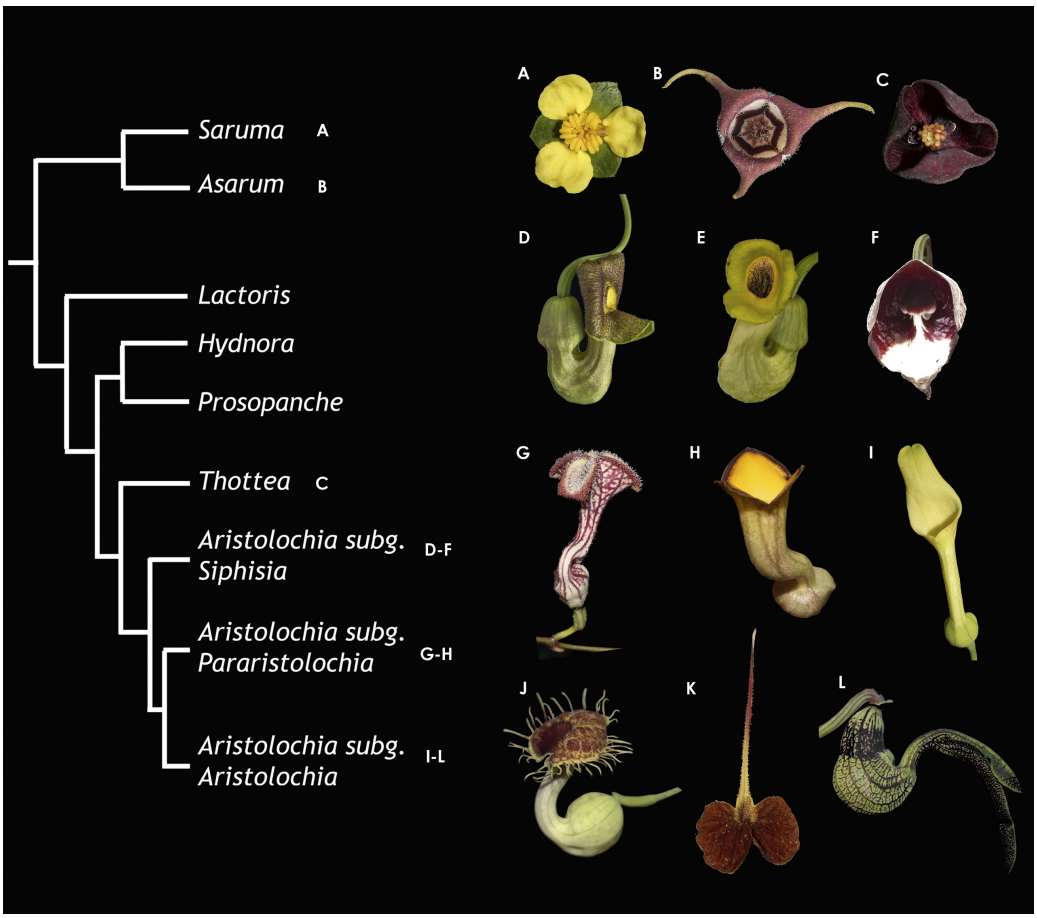


FIGURE 1 | Summarized phylogeny of the Aristolochiaceae with representative photographs for the main lineages. **(A)** *Saruma henryi*. **(B)** *Asarum canadense*. **(C)** *Thottea siliquosa*. **(D)** *Aristolochia macrophylla*. **(E)** *A. manshuriensis*. **(F)** *A. arborea*. **(G)** *A. deltantha*. **(H)** *A. praevenosa*. **(I)** *A. clematitis*. **(J)** *A. fimbriata*. **(K)** *A. lindneri*. **(L)** *A. ringens*.

three sepals in the sole species of *Lactoris (L. fernandeziana)* are light-green (Bernardello et al., 1999; González and Rudall, 2001). Perianth colors in *Thottea* vary from dark purple to brown to red, suffused with yellow or white (Shaiju and Omanakumari, 2010). The highly elaborated, sepal-derived perianth in *Aristolochia,* is exceedingly diverse in terms of color, size, and shape (Wagner et al., 2012, 2014; Pabón-Mora et al., 2020; **Figure 1**). *Aristolochia* sepals are often green in their outer surface and variously colored with purple, red, yellow, or white in their inner surface. Because typical petal features such as colors (other than green), papillae and osmophores are present in *Aristolochia* sepals, these organs are thought to present a transfer of function from petals (Pabón-Mora et al., 2015). Additionally, different colors are displayed forming a vast range of species-specific patterns, including reticulations, streaks, lines, spots or dots that are showier in the inner surface of the limb.

Here we aim to: (1) identify SG6 *R2R3-MYB* homologs, putatively associated with flavonoid production in members of the Aristolochiaceae; (2) reconstruct their evolutionary history across flowering plants, with a sampling focused on non-core eudicots; (3) compare their expression during flower development on selected genera within the Aristolochiaceae; (4) understand the spatio-temporal activation of TFs and enzymes during the development of *Aristolochia fimbriata*; and (5) plot the flavonoid and anthocyanin metabolic pathways for representative members of the Aristolochiaceae.

MATERIALS AND METHODS

Isolation and Phylogenetic Analyses of SG6 *R2R3-MYB* Genes

Canonical SG6 *R2R3-MYB* genes from *Arabidopsis thaliana* (*AtMYB75*, *AtMYB90*, *AtMYB113*, and *AtMYB114*) were used as queries to identify putative homologs in the available Aristolochiaceae transcriptomes (see Pabón-Mora et al., 2015, 2020). The transcriptomes were generated from mixed leaves, flowers, and fruits (when available) for the following species: *Aristolochia arborea, A. clematitis*, *A. deltantha*, *A. lindneri*, *A. macrophylla*, *A. manshuriensis, A. praevenosa*, *A. ringens, Asarum canadense*, *A. europaeum*, *Saruma henryi*, and *Thottea siliquosa*. The *Arabidopsis thaliana* sequences were retrieved

from TAIR[1]. All other sequences included in the analysis outside Aristolochiaceae and Brassicaceae were downloaded from public sequence repositories such as NCBI[2], Phytozome[3], OneKP (Leebens-Mack et al., 2019)[4], The Plant Transcription Factor Database (Jin et al., 2016)[5], and our own transcriptomes. Searches were performed using BLASTN (Altschul et al., 1990). Closely related *Arabidopsis R2R3-MYB* subgroups SG4, SG5, SG7, and SG15, were also retrieved and used as outgroups. The selection of several subgroups as outgroups allowed us to identify SG6 orthologs. Homologs included in these analyses are in **Supplementary Table 1**. Newly isolated sequences from this work can be found in GenBank under the accessions MW125647–MW125662 and MW788582–MW788641.

All sequences were compiled using BioEdit[6] where they were cleaned to find the open reading frame and to keep exclusively the coding sequence (CDS) by removing the flanking untranslated regions (UTRs). Nucleotide sequences were aligned using the online version of MAFFT V7 (Katoh et al., 2019) with a gap open penalty of 3.0 and an offset value of 1.0. All other default settings were used without further modification. Maximum Likelihood (ML) phylogenetic analyses were done using IQ-Tree through the W-IQ-TREE portal[7]; Trifinopoulos et al., 2016). The molecular evolution model that best fit the data was calculated using the ModelFinder tool incorporated in IQ-TREE (Kalyaanamoorthy et al., 2017). The Ultrafast Bootstrap (UFBS) of 1000 pseudo-replicas also implemented in IQ-TREE was used to calculate branch support (Hoang et al., 2018). Phylogenetic trees obtained were visualized and edited on FigTree[8] and labeled in Photoshop Illustrator CC 2019. Names of previously reported sequences were kept as published or with the original codes extracted from the databases. Gene names for the homologs isolated in this work were assigned based on their higher similarity to the *AtMYB114* canonical *Arabidopsis* gene, over the other three paralogs. In turn, they have all been named *MYB114-like*. Two analyses are presented, one with a comprehensive outgroup (SG4, SG5, SG7, and SG15) and all SG6 homologs including the monocot *R2R3-MYB* SG6 gene representatives (**Supplementary Figure 1**), and one restricted to SG6 homologs using *Arabidopsis thaliana MYB123* as outgroup (**Figures 2**, **3**).

Protein Sequence Analysis

In order to verify whether the previously reported domains for R2R3-MYB proteins were retained in all angiosperms and specifically if they were found in the Aristolochiaceae protein homologs, a MEME analysis was carried out. For this, the amino acid sequences were permanently translated using BioEdit. The resulting file was then introduced to the Multiple Em for Motif Elicitation (MEME) online server[9] and analyzed using the default parameters and enquiring for the top 20 motifs. The motifs retrieved by MEME are reported according to their statistical significance. Within the given sequences, the MEME suite finds the most statistically significant (low *e*-value) motifs first. All SG6 *R2R3-MYB* sequences used in the phylogenetic analysis were included in this analysis (**Figure 4**). In addition, detailed amino acid alignments for the R2 and R3 domains as well as for the characteristic motif 4 of SG6 were exported from BioEdit (**Figure 5** and **Supplementary Figure 2**).

Expression Analyses by Reverse Transcriptase – PCR (RT-PCR)

Expression of SG6 *R2R3-MYB* homologs was assayed using RT-PCR on dissected perianth parts in different floral developmental stages from four selected species. Namely, *Saruma henryi*, *Asarum canadense* and two members of *Aristolochia* (*A. fimbriata* and *A. manshuriensis*). We chose them as they represent different floral groundplans and include contrasting patterns in the variation range for color display in the Aristolochiaceae, as will be described below.

Saruma henryi flowers were taken in early June 2019 from plants growing outdoors at the Botanischer Garten Dresden (Dresden, Germany), with approximately 12 h daylight and ca. 16°C; floral buds in pre-anthesis of ca. 0.8 cm were dissected, separating the bright yellow petals from the green sepals (**Supplementary Figure 3A**).

Asarum canadense flowers were collected from living collections at the Arnold Arboretum at Harvard University (Roslindale, MA, United States; Plant ID: 204-2006*MASS-A). Sepals were taken at four different stages, as follows: stage S0, when the inner surface of the sepals was still yellow; S1, when purple color was first evident; pre-anthesis, when sepals were most intensely purple; and anthesis, when purple accumulation decreased (**Supplementary Figure 3B**).

Aristolochia fimbriata flowers were taken from plants cultivated under 16 h light and ca 22°C at the Evo-Devo lab (University of Antioquia, Medellín, Colombia). They were dissected in two developmental stages: S6 with completely green perianth and S9 (or pre-anthesis) flowers with pigmented flowers displaying intermixed yellow lines separating dark purple sections in the limb, a mostly purple tube, and a purple spotted predominantly pale-yellow utricle (Stages follow Pabón-Mora et al., 2015). The three perianth portions (utricle, tube and limb) were dissected accordingly (**Supplementary Figure 3C**).

Aristolochia manshuriensis flowers were collected in early May 2019 from living collections at the Arnold Arboretum at Harvard University (Roslindale, MA, United States; Plant ID: 424-87*D), with approximately 10 h daylight and 10°C. Floral buds in two different developmental stages in pre-anthesis, in addition to flowers in anthesis were collected. The perianth at the S2 stage, with completely green/yellow perianth was used without further dissection between limb, tube and utricle, as no differences in color were evident. Conversely, the S9 perianth showed color difference in the inner surface of the

[1]https://www.arabidopsis.org/

[2]https://www.ncbi.nlm.nih.gov/

[3]https://phytozome.jgi.doe.gov/pz/portal.html

[4]https://sites.google.com/a/ualberta.ca/onekp/

[5]http://planttfdb.gao-lab.org/

[6]https://bioedit.software.informer.com/7.2/

[7]http://iqtree.cibiv.univie.ac.at/

[8]http://tree.bio.ed.ac.uk/software/figtree/

[9]http://meme-suite.org/

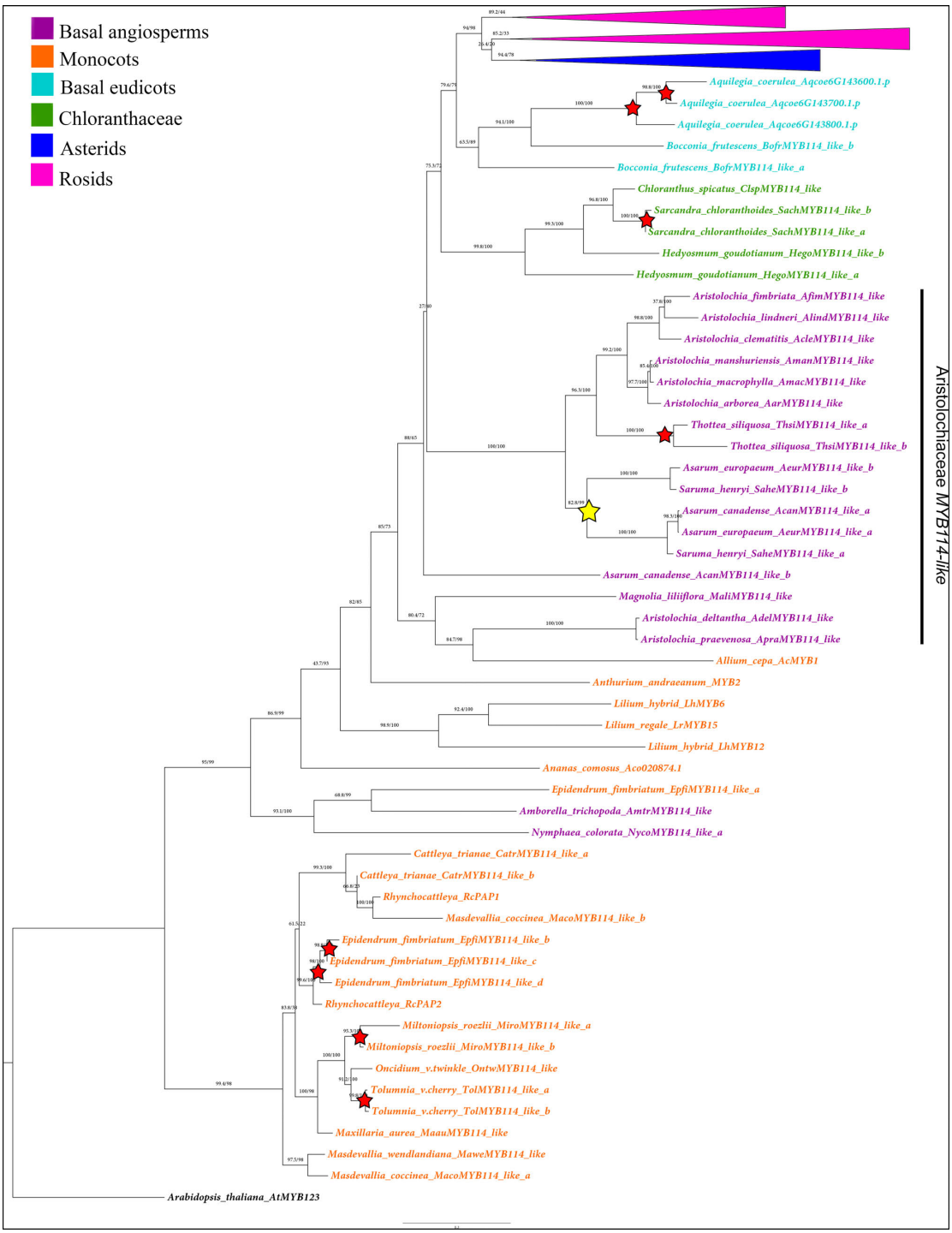


FIGURE 2 | Maximum likelihood analysis of the SG6 *R2R3-MYB* genes with expanded view of the non-core eudicot gene homologs. Yellow star indicates large-scale duplication events in the Aristolochiaceae prior to the diversification of *Asarum* and *Saruma*. Red stars represent species-specific duplication events. Color clades follow the conventions in the top left.

utricle (dark purple), the tube (yellow and purple) and the limb (yellow); these three portions were dissected accordingly. Finally, only the yellow limb was dissected in anthetic flowers (**Supplementary Figure 3D**).

Total RNA from the individual dissected floral portions described above was extracted using TRIsure, following the manufacturer's protocol (Bioline, London, United Kingdom). All RNA extractions were performed right after tissue collection

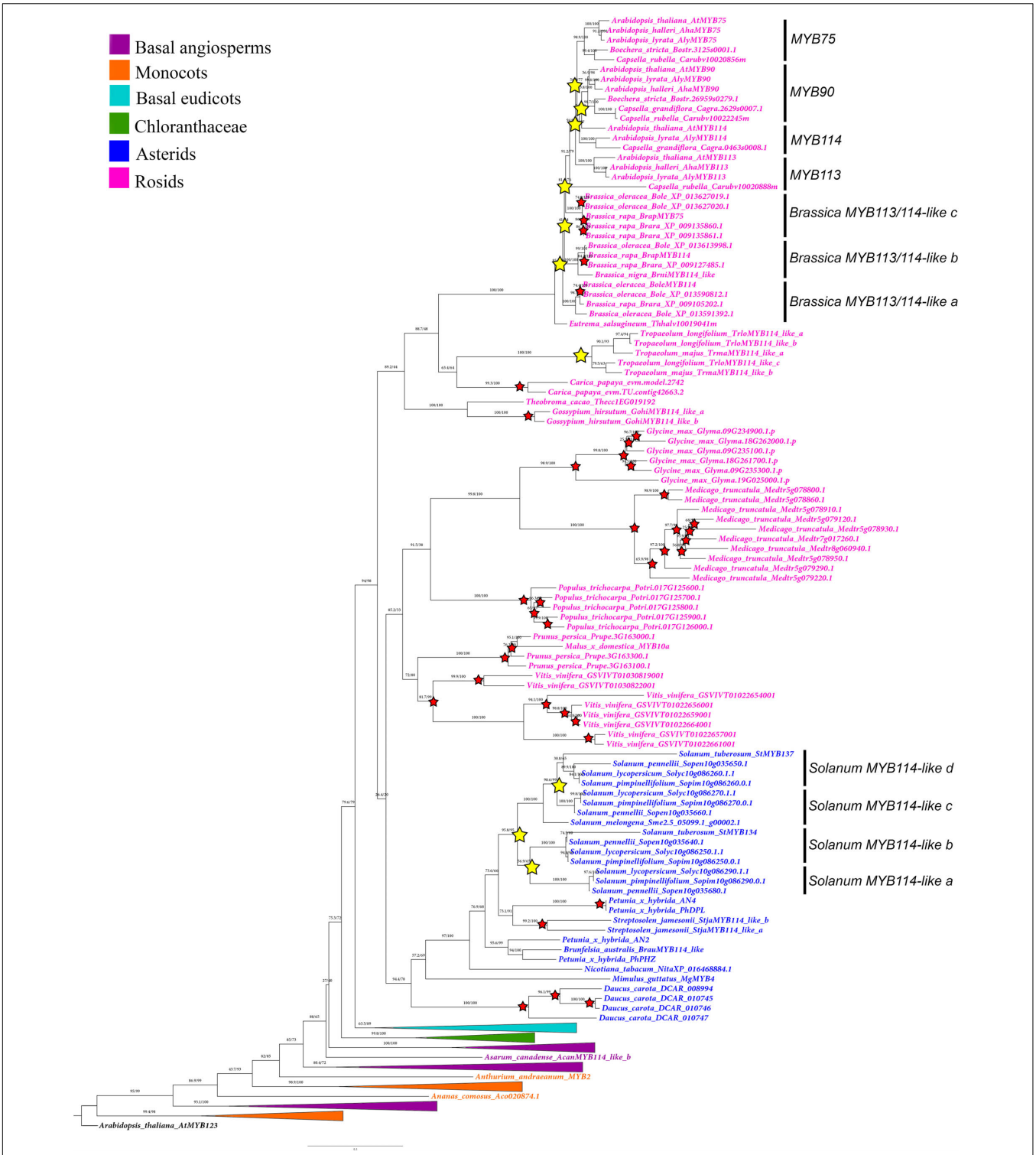


FIGURE 3 | Maximum likelihood analysis of the SG6 *R2R3-MYB* genes with expanded view of the core-eudicot gene homologs. Yellow stars indicate large-scale duplication events in *Solanum* and Brassicales. Red stars represent species-specific duplication events. Color clades follow the conventions in the top left.

in liquid nitrogen. RNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific). Total RNA obtained was treated with DNAseI (Roche, Switzerland) to remove genomic DNA contamination. cDNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, United States). One μg of RNA was used in every reaction

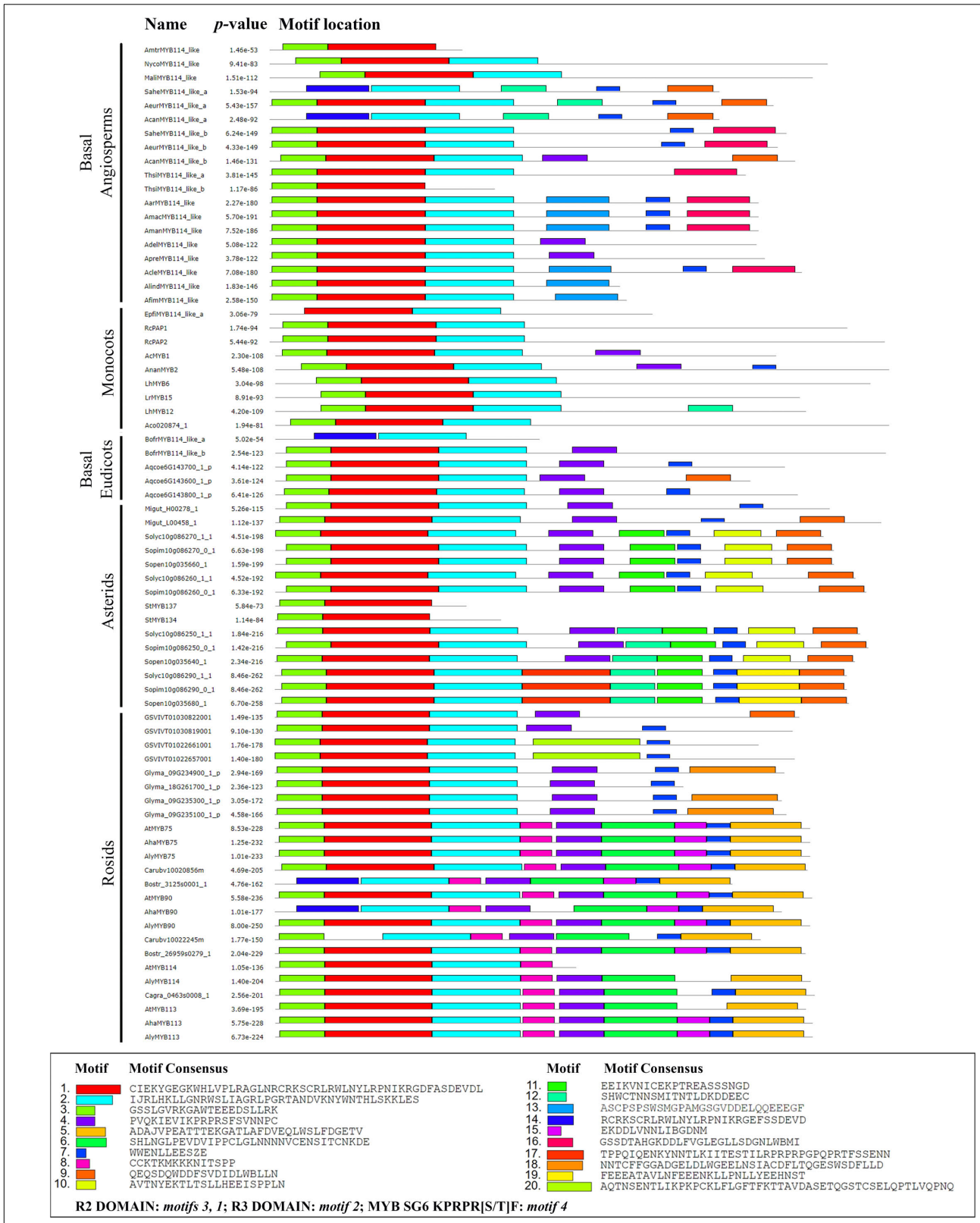


FIGURE 4 | MEME analysis of selected SG6 R2R3-MYB proteins from core and non-core eudicots. Motifs found are indicated by color boxes. The sequence corresponds to the consensus, that is the amino acids occurring at higher rates in the motif. The R2 domain corresponds to motifs 1 and 3; R3 domain corresponds to motif 2. For gene codes, see **Figures 2**, **3** and **Supplementary Table 1**.

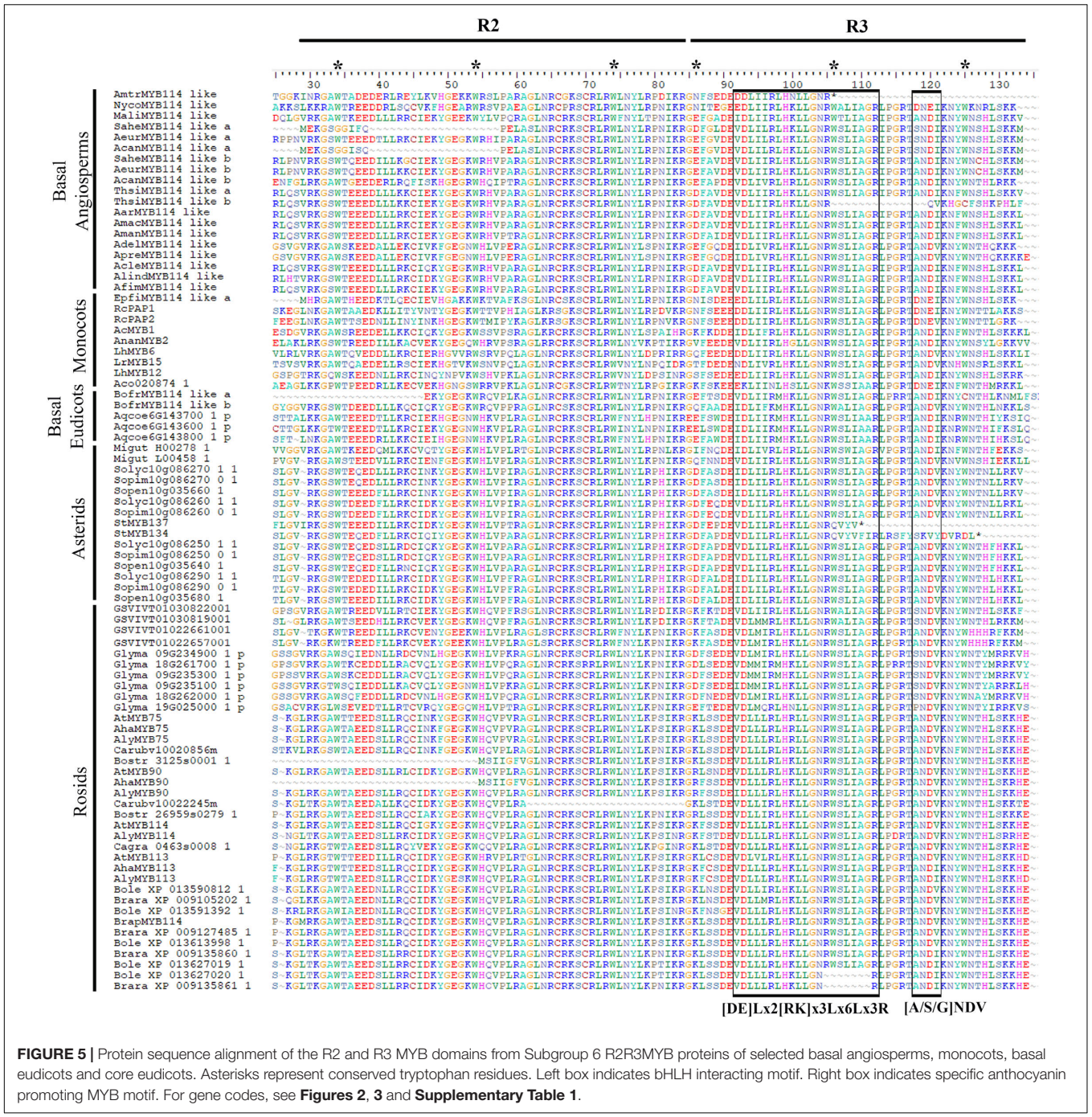


FIGURE 5 | Protein sequence alignment of the R2 and R3 MYB domains from Subgroup 6 R2R3MYB proteins of selected basal angiosperms, monocots, basal eudicots and core eudicots. Asterisks represent conserved tryptophan residues. Left box indicates bHLH interacting motif. Right box indicates specific anthocyanin promoting MYB motif. For gene codes, see **Figures 2**, **3** and **Supplementary Table 1**.

carried, incorporating oligodT primers and following the manufacturer's protocol. PCR was performed using 1 µl of the undiluted cDNA previously obtained and specific primers designed for the SG6 *R2R3-MYB* homolog genes identified in each species (**Supplementary Table 2**). Standardization of melting temperatures and cycles was done in order to determine the amplification reaction peak and saturation. For all species, the thermal cycling regime consisted of one initial step at 95°C for 5 min, 30 amplification cycles repeating the three step: 95°C for 30 s, Tm for 30 s, and 72°C for 50 s, and a final extension step at 72°C for 10 min. Melting temperatures (Tms) were as follows: for *Saruma henryi* PCRs, 48°C; for *Aristolochia fimbriata* and *Asarum canadense* PCRs, 54°C; and, for *A. manshuriensis* PCRs, 56°C. Experiments were carried in a MultiGene OptiMax thermocycler (Labnet International, Edison, NJ, United States). PCR products were run in 1.5% agarose gels with 1X TAE, stained with ethidium bromide. A *MyGel mini* (Accuris instruments, Edison, NJ, United States) electrophoresis camera was used, and results were visualized and digitally photographed using a Whatman Biometra

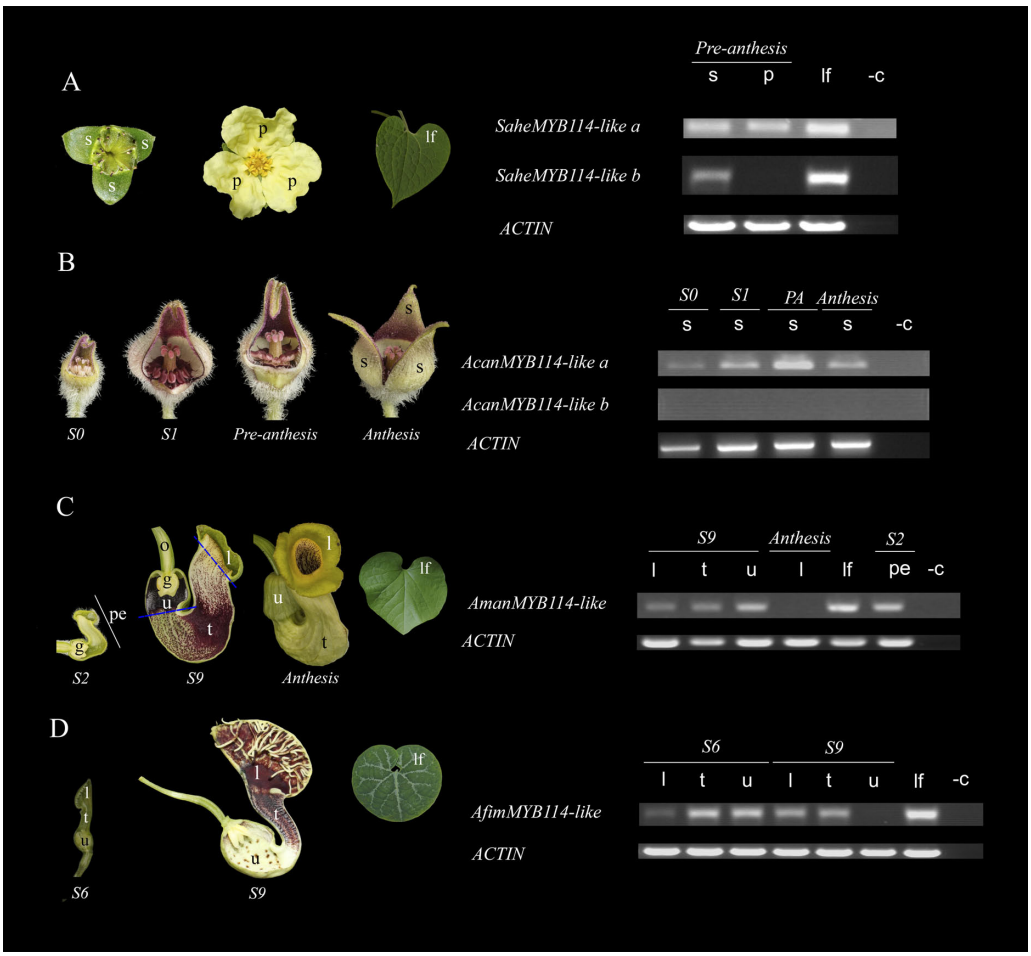


FIGURE 6 | Expression of SG6 *R2R3-MYB* genes in **(A)** *Saruma henryi*. **(B)** *Asarum canadense*. **(C)** *Aristolochia manshuriensis*. **(D)** *A. fimbriata*. *ACTIN* was used as a positive control. *l*, limb; *lf*, leaf; *p*, petal; *pe*, perianth; *s*, sepal; *t*, tube; *u*, utricle; -c, amplification reaction without cDNA (negative control).

BioDocAnalyzer (Göttingen, Germany). Original electrophoresis results are shown without brightness or contrast modifications (**Figure 6**). Quantitative data was obtained by Image J analyses of pixel intensity compared to *ACTIN* expression for all samples (**Supplementary Figure 4**). This was done following Wittall et al. (2006).

Differential Gene Expression by RNA-Seq in *Aristolochia fimbriata*

De novo transcriptomes from *Aristolochia fimbriata* were generated as follows. Each transcriptome was obtained from three independent biological replicates of the dissected portions of the sepal-derived perianth, namely, the limb, the tube, and the utricle, at S6 and S9. The experiment was conducted in order to assess differentially expressed genes (DEGs) between the three portions of the perianth at two different developmental stages. Total RNA from the two dissected floral stages was extracted using TRIzol reagent (Invitrogen). The RNA-seq experiment was conducted using the Truseq stranded mRNA library construction kit (Illumina) and sequenced on a NovaSeq 6000 system reading 100 bp, paired-end reads. Read cleaning was performed with PRINSEQ-LITE (v0.20.4) available at http://printseq.sourceforge.net, with a quality threshold of Q30 at both ends and only keeping those longer that 70 bases after quality trimming. Contig assembly was computed using the Trinity package following default settings. Transcriptome assembly was performed for each perianth portion, in both developmental stages (**Supplementary Table 3**). In addition, a combined global transcriptome from all experiments was assembled as a reference with the following metrics: total assembled bases: 85.608.833 bp; total number of contigs (>101 bp): 118.941; average contig length: 719 bp; contig N50: 14.432 sequences ≧ 1.823 bp; contig N75: 31.828 sequences ≧ 746 bp; contig GC%: 42.71%.

To estimate the relative abundance of the assembled contigs, cleaned reads were mapped against the *de novo* assembled dataset implementing the algorithm Kallisto v.0.46.0 with default settings[10]. Kallisto quantifies transcript expression normalizing the relative abundance of each contig/transcript using the transcript per million (TPMs) metrics (Bray et al., 2016;

[10]https://pachterlab.github.io/kallisto/

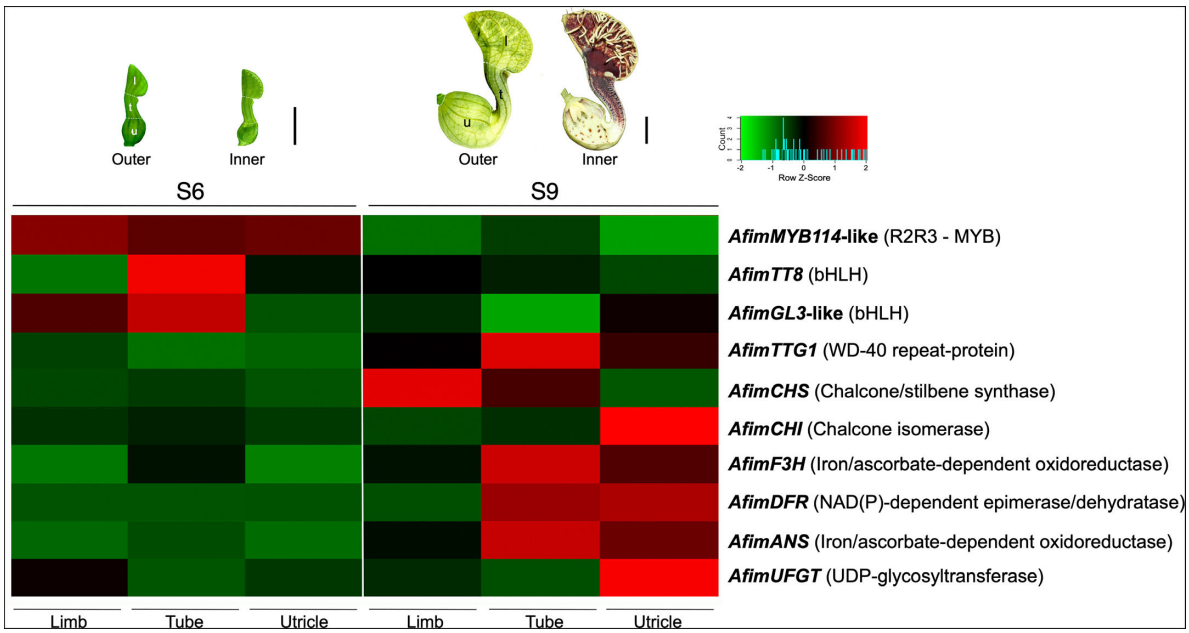


FIGURE 7 | Expression analysis of genes involved in flavonoid production in *Aristolochia fimbriata*. The heatmap was generated based on normalized RNA-seq data. Ten genes were analyzed, six structural genes and four regulatory genes involved in the flavonoid biosynthetic pathway. Two different developmental stages (S6 and S9) and three portions of the perianth (limb, tube, and utricle) were compared. The color codes indicate upregulated (red) and downregulated (green).

Supplementary Table 4). Homologs for the bHLH and the WD40 families as well as for all enzymes were identified by reciprocal BLASTN searches. The homology for the transcription factors was confirmed by phylogenetic analyses (**Supplementary Figures 5–7** and **Supplementary Tables 5, 6**). The relative abundance of *AfimMYB114*-like, *AfimTT8, AfimGL3-like*, *AfimTTG1, AfimCHS, AfimCHI, AfimF3H, AfimDFR, AfimANS,* and *AfimUFGT* transcripts was used to identify their expression level in each portion of the *Aristolochia fimbriata* perianth. This expression was calculated for the six generated transcriptomes corresponding to the utricle, the tube, and the limb at two different developmental stages (S6 and S9). Expression data from each sample was used to construct the heatmaps using the Shinyheatmap program (**Figure 7**)[11] (Khomtchouk et al., 2017).

Gene Annotation and Pathway Mapping

Genes of the flavonoid and anthocyanin biosynthetic pathway were plotted using the KAAS tool of the KEGG database (Kanehisa and Goto, 2000). Previously obtained transcriptomes for different Aristolochiaceae species were translated to amino acid sequences and partitioned in several files due to size constrains. TransDecoder software[12] was used for transcriptome translation. Protein sequences were submitted to the KEGG website[13]. The resulting analyses include the mapping of all enzymes found for the flavonoid and anthocyanin pathways for each species (**Figure 8**).

[11]https://github.com/Bohdan-Khomtchouk/shinyheatmap
[12]https://github.com/TransDecoder/TransDecoder/wiki
[13]https://www.genome.jp/kegg/

RESULTS

Subgroup 6 *R2R3-MYB* Gene Evolution

First, we wanted to assess if the distantly related Aristolochiaceae had homologs from the SG6 *R2R3-MYB* genes reported in *Arabidopsis thaliana* (Brassicaceae) as they are central components of the MBW complex. The directed search using the SG6 *R2R3-MYB* canonical genes that integrates the MBW complex in *Arabidopsis thaliana* to assess Aristolochiaceae homologs resulted in 16 *R2R3-MYB* putative hits. In order to reconstruct the evolution of the SG6 *R2R3-MYB* genes and to verify homology, other genes from representative angiosperms were also retrieved from online databases and our own transcriptomes. Altogether, this sampling resulted in a total of 155 sequences. Namely, 3 gene sequences from early divergent angiosperms (ANA), 16 from Aristolochiaceae, 23 from monocots, 10 from basal eudicots and Chloranthaceae, and 103 sequences from core eudicots (**Supplementary Figure 1** and **Figures 2**, **3**). A first phylogenetic analysis with a comprehensive outgroup (SG4, SG5, SG7, and SG15) and all SG6 homologs including the monocot *R2R3-MYB* SG6 gene representatives (**Supplementary Figure 1**) allowed us to confirm SG6 homologs with a bootstrap support (BS = 90). A second phylogenetic analysis excluded the closely related subgroups to concentrate only in the SG6 genes. Following the first analysis *AtMYB123* was used as outgroup (**Figures 2**, **3**). Newly identified genes were named as *MYB114-like,* as they resulted to be more similar in protein sequence to AtMYB114 than to any of the other three canonical *Arabidopsis* copies AtMYB75, AtMYB90 or AtMYB113. Our goal was to use a name that indicates their

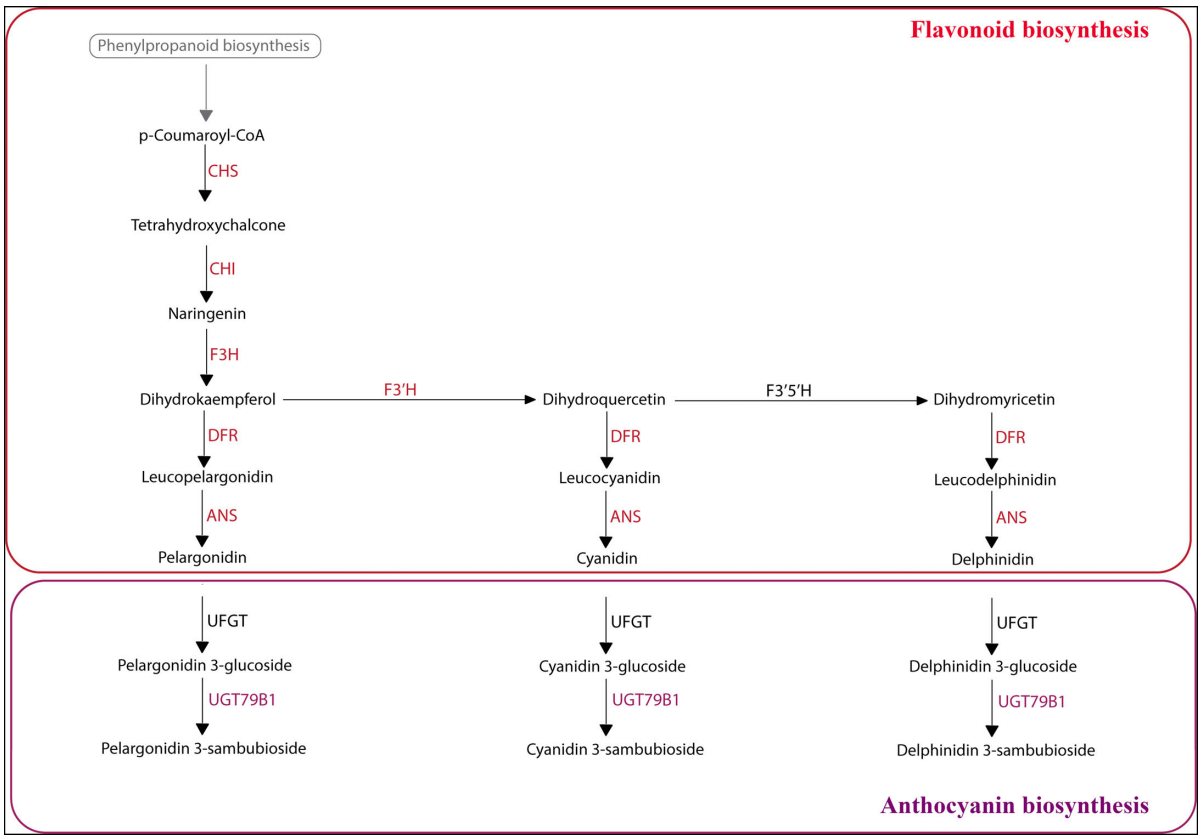


FIGURE 8 | KEGG analysis of selected Aristolochiaceae species using predicted peptides. Summary flavonoid/anthocyanin biosynthetic pathway based on KEGG results. Colored enzyme names (in red and purple) represent those enzymes present in the peptide data from the mixed reference transcriptome in most analyzed species. For exceptions, see results. In black are the enzymes that were not found. ANS, Anthocyanidin synthase; CHI, Chalcone isomerase; CHS, Chalcone synthase; DFR, Dihydroflavonol 4-reductase; F3H, Flavanone-3-hydroxylase; F3′H, Flavonoid-3′-hydroxylase; F3′5′H, Flavonoid-3′5′-hydroxylase; UFGT, Anthocyanidin 3-O-glucosyltransferase; UGT79B1, Anthocyanidin 3-O-glucoside 2‴-O-xylosyltransferase.

affinities to SG6 and the corresponding canonical *Arabidopsis* genes. All other genes in the tree retain the previously published names or codes.

The ML analysis of the SG6 *R2R3-MYB* genes is mostly consistent with the phylogenetic relationships of the major lineages sampled (**Supplementary Figure 1** and **Figures 2**, **3**). The exceptions are found in monocot homologs identified as SG6 homologs, several of which resulted as sister group to all other *R2R3-MYB SG6* genes (**Figure 2** and **Supplementary Figure 1**). For the most part SG6 homologs reconstruct the evolution of angiosperms, having early diverging SG6 homologs as sister to Chloranthaceae and eudicot genes, and within eudicots representative subclades of asterid and rosid sequences (**Figures 2**, **3**). Interestingly, the reconstruction of the SG6 *R2R3-MYB* gene evolution shows a larger number of local or species-specific duplications than large-scale duplications across angiosperms. In the Aristolochiaceae, the SG6 *R2R3-MYB* genes are mostly found as single copy genes and have undergone one duplication prior to the diversification of *Saruma* and *Asarum* resulting in the *MYB114-like a* and *MYB114-like b* clades (Bootstrap support BS = 84; **Figure 2**). However, one of the *A. canadense* copies (hereafter named *AcanMYB114-like*) comes out in an odd position as sister to all other Aristolochiaceae sequences, rather than nested in any of the two *MYB114-like* clades mentioned above. An additional species-specific duplication was found in *Thottea siliquosa* (BS = 100; **Figure 2**). The sequences from *T. siliquosa* are sister to those from species of *Aristolochia* subg. *Siphisia* and *Aristolochia* subg. *Aristolochia*, mirroring the phylogenetic sister-group relationships of these two genera. The sequences from species belonging to *Aristolochia* subg. *Pararistolochia* have a large number of non-synonymous changes and occupy an odd position as they cluster with one monocot sequence from *Allium cepa* (**Figure 2**). Nevertheless, in all analyses, they fall as members of the SG6 subgroup (**Supplementary Figure 1** and **Figure 2**).

Additional large-scale duplications found in this gene linage occur in particular core eudicot groups. Some of them are linked to the diversification of the genus *Solanum* (Solanaceae), resulting in the *SolanumMYB114-like a* (BS = 100), *SolanumMYB114-like b* (BS = 100), *SolanumMYB114-like c* (BS = 100), and *SolanumMYB114-like d* (BS = 89) clades (**Figure 3**). Independent duplications have occurred in the Brassicales for Brassiceae (including *Brassica*) and Camelinae (including *Arabidopsis*) members (**Figure 3**). Three duplications have occurred in the

Camelinae resulting in the *MYB75* (BS = 99), *MYB90* (BS = 71), *MYB113* (BS = 100), and *MYB114* (BS = 100) clades, which include the canonical *Arabidopsis* paralogs. There are two additional independent duplications in Brassiceae which we have labeled *Brassica MYB113/114-like a* (BS = 100), *MYB113/114-like b* (BS = 100) and *MYB113/114-like c* (BS = 100). Perhaps more inclusive sampling from more diverse species across the Brassicales will allow to assess the exact time point of these duplication events.

Additional species-specific duplications have occurred in *Aquilegia coerulea* (Ranunculaceae), *Bocconia frutescens* (Papaveraceae), *Carica papaya* (Caricaceae), *Daucus carota* (Apiaceae), *Glycine max* (Fabaceae), *Gossypium hirsutum* (Malvaceae), *Hedyosmum goudotianum* (Chloranthaceae), *Lilium 'hybrid'* (Liliaceae), *Medicago truncatula* (Fabaceae), *Mimulus guttatus* (Phrymaceae), *Nelumbo nucifera* (Nelumbonaceae), *Petunia hybrida* (Solanaceae), *Populus trichocarpa* (Salicaceae), *Prunus persica* (Rosaceae), *Sarcandra chloranthoides* (Chloranthaceae), *Solanum tuberosum* (Solanaceae), *Streptosolen jamesonii* (Solanaceae), *Tropaeolum longifolium* (Brassicaceae), and *Vitis vinifera* (Vitaceae) (**Figures 2**, **3**).

Comparative Analysis of the Subgroup 6 R2R3-MYB Protein Sequences

Next, we wanted to verify if the typical R2 and R3 domains were present across all homologs and test for the presence of other conserved motifs that could point to conserved functions in distantly related plant lineages. The *R2R3-MYB* genes present a highly conserved N-terminal R2R3 domain that binds DNA. We found that the R2 domain is recovered in the motifs 1 and 3, while the R3 domain is recovered in motif 2 in our analysis (**Figures 4**, **5**). This was verified in the ScanProsite database[14] where motifs 1, 2 and 3 correspond to DNA binding HTH MYB-type domain. Interestingly, motifs 1, 2 and 3 comprising the R2R3 domains are present in most homologs analyzed (**Figure 5**): The exceptions are motif 1 and 3, lacking in the *Saruma henryi* SaheMYB114-like a, the *Asarum canadense* AcanMYB114-like a and the *Bocconia frutescens* BofrMYB114-like a. On the other hand, motif 2 is lacking in the *Thottea siliquosa* ThsiMYB114-b and the *Amborella trichopoda* AmtrMYB114-like homolog as well as in both *Solanum tuberosum* homologs StMYB134 and StMYB137 (**Figure 4**). These cases likely represent variants with deletions complicating motif recovery by MEME, because visual inspection in the amino acid alignments do show partial regions retained (**Figure 5**).

Motif 4 (KPRPRS/TF) which has been regarded as predictive of SG6 homologs is in fact present in most eudicot sequences, but in early diverging angiosperms can only be found intact in AdelMYB114-like, ApraMYB114-like and AcanMYB114-b, while there are several non-synonymous substitutions in the rest of the taxa sampled (KLPNSV; **Supplementary Figure 3**). Monocot homologs also lack an intact SG6 predictive motif and present a divergent RPQPR/K. Other motifs identified by MEME seem to be characteristic of some groups. For instance, motifs 5, 6, 8, and 15 can help recognize R2R3-MYB proteins from Brassicales (**Figure 4**). Motifs 9, 10, and 19 are characteristic of several proteins in the Solanales. Finally, some motifs seem to be species-specific, as motif 18 appears only in *Glycine max* homologs and motif 20 is only present in *Vitis vinifera* proteins. None of the new motifs identified has any reference functions; however, these conform shared protein sequences that will have to be tested in the future for specific roles.

[14]https://prosite.expasy.org/scanprosite/

Expression Analysis of the Subgroup 6 *R2R3-MYB* Genes in Selected Aristolochiaceae

The next step in our study was to analyze the expression patterns of the SG6 *R2R3-MYB-like* genes in different Aristolochiaceae species. For this, the following four Aristolochiaceae species were selected based on the color variation of their perianth: *Saruma henryi*, with a biseriate perianth formed by three green sepals and three yellow petals; *Asarum canadense*, with a uniseriate perianth formed by three dark purple fused sepals; *Aristolochia fimbriata,* with a uniseriate perianth formed by three sepals with an inner surface mainly purple and yellow; and *A. manshuriensis* with a uniseriate perianth formed by three sepals with an inner surface with mainly green, dark purple and yellow tones (**Figure 6**). Whenever possible, different developmental stages and perianth parts were sampled (see section "Materials and Methods"). *ACTIN* was used as a positive control in all samples.

In *Saruma henryi*, the SG6 *R2R3-MYB-like* gene *SaheMYB114-like-a* is broadly expressed in sepals, petals and leaves, but *SaheMYB114-like-b* is restricted to the green sepals and the leaves. No expression of this homolog was detected in the yellow petals. In *A. canadense*, *AcanMYB114-like-a* is found in sepals through all developmental stages, with a peak of gene expression, which coincides, with a visual peak of anthocyanin accumulation in the perianth during pre-anthesis. No expression was found for *AcanMYB114-like-b*. In *A. manshuriensis*, *AmanMYB114-like* is expressed early in the light green perianth prior to any purple accumulation in its inner surface at S2. The same broad expression is found for *AmanMYB114-like* at S9 in the green limb, the reddish tube, and especially in the dark purple utricle. Expression of *AmanMYB114-like* is lacking in the yellow limb at anthesis. Importantly, *AmanMYB114-like* is actively expressed in the green leaves. Finally, in *A. fimbriata*, *AfimMYB114-like* is present in the limb, tube, and utricle at S6, by the time the inner perianth surface is still green throughout. Later, in flowers at S9, the expression of *AfimMYB114-like* becomes restricted only to the purplish limb and tube. Like its homolog, *AfimMYB114-like* is expressed in the green leaves (**Figure 6**).

Differential Expression Analysis in the *Aristolochia fimbriata* Perianth

Homologs of all members forming the MBW complex were identified in the Aristolochiaceae (**Supplementary Figures 5–7**). Comparative expression levels of the regulatory (*AfimMYB114-like, AfimTT8, AfimGL3,* and *AfimTTG1*) and structural genes (*AfimCHS, AfimCHI, AfimDFR, AfimF3H, AfimANS,* and

AfimUFGT) were tested in the limb, the tube, and the utricle at S6 and S9 floral developmental stages (**Figure 7**). Perianth at S6 flowers is completely green. In contrast, S9 flowers display differential color patterns in the perianth. The limb's background is dark purple with yellow longitudinal and transversal stripes, the tube has dark purple and light yellow longitudinal stripes, and the utricle is mostly light yellow with a few dark purple spots scattered through it.

Most of the regulatory genes (*AfimMYB114-like*, *AfimTT8,* and *AfimGL3*) begin their expression even before the acquisition of the purple color in the perianth at S6. Importantly, their highest accumulation coincides with the early onset of purple coloration in the tube. At S6, none of the structural genes (i.e., *AfimCHS, AfimCHI, AfimDFR, AfimF3H, AfimANS,* and *AfimUFGT*) seem to be particularly active. However, most of these genes increase in expression at S9 concomitant with the reduced levels of the regulatory genes. Among structural genes, only *AfimCHS* seems to be highly expressed in the limb, while all others concentrate in the tube or the utricle (**Figure 7**). Interestingly, *AfimTTG1* was the only transcription factor of the putative MBW complex found to be highly expressed at the S9 developmental stage.

Comparative Mapping of the Anthocyanin Biosynthetic Pathway Across Aristolochiaceae

Finally, we wanted to map our reference transcriptomes generated from mixed leaves, flowers and fruits to the flavonoid and anthocyanin pathways from the KEGG database, in order to see which transcription factors were present in selected Aristolochiaceae. First, the flavonoid pathway was analyzed. For *Saruma henryi*, *Asarum canadense, Asarum europaeum*, *Thottea siliquosa*, *Aristolochia deltantha*, *A. lindneri*, *A. macrophylla*, *A. manshuriensis*, and *A. praevenosa*, all the core flavonoid biosynthesis enzymes were present, except for the flavonoid 3',5'-hydroxylase (CYP75A) which did not have a match in any of the peptides provided for any species (**Figure 8**). We noticed two additional enzymes lacking specific matches in two *Aristolochia* species, namely, the bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase (DFR) and the anthocyanidin synthase (ANS) enzymes were missing from the *A. arborea* peptide dataset, and the same anthocyanidin synthase (ANS) could not be found in the *A. clematitis* dataset.

Next, the anthocyanin biosynthetic pathway was evaluated. A single enzyme of this pathway, the 3-O-glucoside 2‴-O-xylosyltransferase (UGT79B1) enzyme is present in all Aristolochiaceae evaluated, except in *A. arborea* (**Figure 8**). One interesting case is the UFGT enzyme, which performs flavonoid glycosylation and thus is critical for final anthocyanin production. This enzyme did not find predicted peptide matches in any of the species evaluated. However, a directed search using the RNA-seq data from *A. fimbriata* provided a match for this enzyme. In turn, predicted peptides may be less reliable than BLASTN searches for automatizing pathway predictions.

DISCUSSION

R2R3-MYB Gene Diversification Is Likely Driven by Tandem Duplication Events in Core Eudicots

In *Arabidopsis thaliana,* four *R2R3-MYB* genes involved in the anthocyanin biosynthesis pathway have been reported, namely, *AtMYB75*, *AtMYB90*, *AtMYB113*, and *AtMYB114* (Stracke et al., 2001). All SG6 *R2R3-MYB* genes control early anthocyanin biosynthesis in vegetative tissues (Dubos et al., 2010) and overexpression of *MYB75* and *MYB90* by activation tagging can result in darker leaves and purple petals in *Arabidopsis* (Borevitz et al., 2000). Similarly, overexpression of the *Eutrema salsugineum EsMYB90* gene in tobacco and *Arabidopsis* results in anthocyanin accumulation and upregulation of structural genes of the flavonoid biosynthesis pathway (Qi et al., 2020). Other studies in red-skinned pear (*Pyrus bretschneideri*) demonstrated that an *R2R3-MYB* homolog, *PyMYB114*, is responsible for anthocyanin biosynthesis in the red fruit skin (Yao et al., 2017). Similarly, the overexpression of *AtMYB75* induces anthocyanin production in *Solanum lycopersicum* starting in the seedling until the development of stems and leaves (Zuluaga et al., 2008). Likewise, an *AtMYB113* homolog in *Solanum melongena, SmMYB113*, induces anthocyanin accumulation by binding to the promoter of the chalcone isomerase (SmCHI) and dihydroflavonol 4-reductase (SmDFR) enzymes (Li et al., 2017). Outside eudicots the SG6 *R2R3-MYB* homologs have been less studied. The *RcPAP1* and *RcPAP2 Cattleya* hybrids homologs do activate the anthocyanin synthesis pathway in the flowers (Li et al., 2020). Altogether, the present data on gene homologs belonging to the SG6 *R2R3-MYB* genes suggest that they perform key early roles in the control of the anthocyanin pathway in different angiosperms and in different plant organs. However, to date no comprehensive phylogenetic framework including the functionally analyzed *SG6 R2R3-MYB* core eudicot homologs is available, and it is less clear if their roles outside of eudicots and monocots are maintained.

Our analysis shows that many model core-eudicots including members of the Solanaceae and Brassicaceae have undergone local duplication events (**Figure 3**). For instance, we identified three duplications exclusive to *Solanum* yielding four (a–d) *SolanumMYB114-like* clades (**Figure 3**). Interestingly, *Solanum tuberosum*, a well-known polyploid, has only two gene copies, compared to the diploid *S. lycopersicum,* which has four copies, one on each of the *SolanumMYB114-like* clades. This reinforces the hypothesis that the evolution of *Solanum MYB* genes has been driven by tandem duplications and not by whole-genome duplication events (Sun et al., 2019).

Similar local duplications were identified in Brassicales. Three duplications resulted in the diversification of the *MYB90*, *MYB75* and *MYB113,* and *MYB114* clades in *Arabidopsis* and close relatives belonging to the Camelinae (Guo et al., 2017). Independent additional Brassiceae specific duplications resulted in three major clades (*MYB113*/*MYB114 a, b,* and *c*) and additional species-specific duplications in *Brassica* species are recorded. In turn, our results show that *B. oleracea* and *B. rapa*

have more gene copies than *Arabidopsis*. This condition could be related to independent genome duplication events associated with the two genera (Wang et al., 2015).

Local duplications are extremely common in other basal and core eudicots evaluated. We found that 17 of the total species evaluated presented species-specific duplications. Such tandem duplications had also been identified in *Aquilegia coerulea*, *Medicago truncatula*, *Populus trichocarpa*, and *Vitis vinifera*, suggesting that small-scale duplications have been important for the expansion of this gene subfamily (Wilkins et al., 2009; Du et al., 2015).

Conversely, the SG6 *R2R3-MYB* genes in monocots and early divergent angiosperms show few to none local duplications. We speculate that tandem duplications are less frequent or that strong selection can be acting upon gene copies, limiting the occurrence or the retention of duplicates. Our ML analysis points to a single duplication in the Aristolochiaceae that predates the *Asarum/Saruma* diversification, which results in the *MYB114-like a* and *b* clades, and one species-specific duplication in *Thottea siliquosa* (**Figure 2**). All remaining Aristolochiaceae and other magnoliids sampled have single copy SG6 *R2R3-MYB* genes. Gene duplications predating the *Asarum/Saruma* diversification have also been detected in the *class II TCP CIN2* genes, involved in the regulation of cell division and floral patterning (Pabón-Mora et al., 2020). It has been previously reported that *Asarum* and *Saruma*, the two members of the subfamily Asaroideae, have a genome size about ten times larger than in species of *Aristolochia* (Bliss et al., 2013). This suggests that gene copy number could be related to larger genome sizes due to whole-genome duplication (WGD) events. Less is known about the genome size of *Thottea siliquosa*, but *class II TCP CIN3* genes are also duplicated in this species, perhaps pointing to unexplored tandem repeats or WGD events occurring in this taxon (Pabón-Mora et al., 2020).

Unusual Domains May Explain Divergent Phylogenetic *R2R3-MYB* Gene Placement

Our analysis of the R2 and R3 domains found in the N-terminal portion of the MYB-R2R3 proteins focused on the three spaced tryptophan residues that form a hydrophobic cluster critical for their function. These tryptophan residues were observed in all the sequences analyzed in this work. As reported by Du et al. (2015), the first residue of the R3 domain was seen to be either phenylalanine (F) or isoleucine (I). The latter two helices of each domain form the HTH structure (helix-turn-helix) and the third is important in the DNA interactions (Wang et al., 2015). The R2 and R3 domains, as reported by Stracke et al. (2001), correspond to motifs 1, 2, and 3 found in our MEME analysis. In turn, it is not surprising that these highly conserved domains with DNA binding function are found in most homologs (**Figure 4**). For the eudicot anthocyanin-promoting MYBs, Liu et al. (2016) report the motif [A/S/G]NDV located inside the R3 domain. This work also reports the bHLH interacting motif in the conserved region of the R3 domain ([DE]Lx2[RK]x3Lx6Lx3R) (**Figure 5**).

Some authors have reported specific motifs for the SG6 R2R3-MYB proteins. Stracke et al. (2001) reported the motif KPRPR[S/T]F at the C terminal portion. This domain is located inside motif 4 from the MEME analysis, and it is present almost intact in eudicots but has dramatic variation in monocots (RPQPR) and basal angiosperms (KPNLC; **Supplementary Figure 3**). Importantly, in the Aristolochiaceae, motif 4 was only found intact in AcanMYB114-like b (KPQPRT), ApraMYB114-like and AdelMYB114-like (KPKPRAL) proteins. This is particularly interesting since all three homologs do not cluster with the other Aristolochiaceae homologs in our phylogenetic hypothesis, suggesting that the retention of motif 4 is rare in Aristolochiaceae. Conversely, motif 7 seems to be more deeply conserved across SG6 R2R3-MYB proteins and can serve hereafter to identify members of this group more easily in non-model species (**Figure 4**). Available functional data does suggest, however, that the canonical SG6 motif KPRPR[S/T]F from eudicots may not be indispensable for the role of these transcription factors in anthocyanin synthesis. At least in monocots the contribution of SG6 R2R3 MYB proteins to purple color in the lip occurs independently of the modifications in the motif (RPMVIR in RcPAP1 and RTKAIR in RcPAP2; **Supplementary Figure 2**; Li et al., 2020).

Expression of *R2R3 MYB* Genes Is Correlated With Anthocyanin Accumulation and Floral Color Patterning

Anthocyanins are best known for conferring different colors in plants, such as red, purple, and blue. Flavonoid biosynthesis enzymes are typically distinguished into two groups: the early biosynthesis genes (EBGs) and the late biosynthesis genes (LBGs) (Fang et al., 2019). *R2R3-MYB* proteins can directly regulate early stage enzymes (SG7 *R2R3-MYBs*), whereas MBW complexes activate later biosynthetic steps (Dubos et al., 2008). These complexes include a R2R3-MYB protein (usually a SG6 protein), a bHLH protein and a WD40 protein, which is not catalytic (Li, 2014). In *Arabidopsis*, the WD40 TF homolog is AtTTG1. The bHLH TFs include AtTT8, AtGL3, and AtEGL1, which have partially redundant functions. All SG6 *R2R3-MYB* factors, namely AtMYB75, AtMYB90, AtMYB113, and AtMYB114 participate in the MBW complex for anthocyanin pathway activation in *Arabidopsis*. Their ectopic expression increases anthocyanin production when associated with bHLH and AtTTG1 (WD40) TFs (Petroni and Tonelli, 2011). Here we wanted to evaluate if the homologs in early divergent angiosperms play similar roles in floral pigmentation.

We found two SG6 *R2R3MYB-like* genes copies for *Saruma henryi* and *Asarum canadense*, and one copy for *Aristolochia manshuriensis* and *A. fimbriata*. Our results on the expression of these genes showed that the SG6 *R2R3-MYB* homologs are expressed in early developmental stages in the sepals, independently of whether they are protective and green at maturity like in *Saruma* or acquire purple color and attract pollinators like in *Asarum* or *Aristolochia*. Such expression may be related to incipient anthocyanin accumulation for

protection against UV radiation and cold temperatures (Sarma and Sharma, 1999; Petroni and Tonelli, 2011). This expression of SG6 *R2R3-MYB* genes in young green floral organs and immature fruits is relatively common. In kiwi (*Actinidia chinensis*) expression of *R2R3-MYB* genes has been observed in green fruits 7 days after anthesis, prior to any notable anthocyanin accumulation (Li et al., 2015). Yellow or white floral parts of *Aristolochia* flowers lack SG6 *R2R3-MYB* gene expression, consistent with low anthocyanin levels (Petroni and Tonelli, 2011). Conversely, dark purple or red perianth organs show high expression levels for these anthocyanin-related *R2R3-MYB* genes. These results indicate that the participation of SG6 *R2R3-MYB* transcription factors as part of the anthocyanin pathway is already in place in Aristolochiaceae. Finally, the differences in expression patterns between the close recent *R2R3-MYB* paralogs in *Saruma* and *Asarum*, suggests that duplications may have resulted in divergent expression of one of the duplicates. At least in *Asarum* one copy *AcanMYB114-like a* retains the role in anthocyanin production, while *AcanMYB114like b* is no longer expressed in the *Asarum* sepals suggesting functional differences after the duplication (**Figure 6**). On the other hand, *SaheMYB114-like a* is expressed in both the green sepals and the yellow petals. This suggests that SG6 *R2R3-MYB* transcription factors in the Aristolochiaceae are more pleiotropic than their equivalents in rosids and asterids.

Mixed purple/red and yellow perianths are relatively common in *Aristolochia*. It has been reported that anthocyanins and carotenoids can occur simultaneously in perianth organs. For example, in some Chilean *Mimulus* species, carotenoids produce the yellow background, while anthocyanins confer a darker coloration in the dorsal petal surface and some spotted areas in the flower throat and central petal (Davies et al., 2012). Mixed yellow and purple perianth in some Aristolochiaceae species may be the result of parallel carotenoid and anthocyanin production in specific floral domains. Thus, addressing how these genes acquire restricted spatio-temporal expression and what other factors can act as negative regulators, is critical to fully understand color patterning in Aristolochiaceae flowers.

Our comparative targeted transcriptomic analysis in two different developmental perianth stages from *A. fimbriata* evaluated four regulatory genes (*AfimMYB114-like*, *AfimTT8*, *AfimGL3*, and *AfimTTG1*) and six structural genes from the flavonoid biosynthetic pathway (*AfimCHS*, *AfimCHI*, *AfimF3H*, *AfimDFR, AfimANS*, and *AfimUFGT*). Phylogenetic analyses confirmed homology for all transcription factors (**Supplementary Figures 5–7**). The RNA-seq expression data confirmed that MYB and bHLH regulatory genes (*AfimMYB114-like*, *AfimTT8*, and *AfimGL3*) are up-regulated in the early developmental stages (S6), while *AfimTTG1* and the structural genes are up-regulated in the later stages of development (S9) (**Figure 7**). Both, the RT-PCR and the RNA-seq data point to upregulation of this pathway at S6 prior to any evident purple color accumulation. Importantly, the SG6 *R2R3-MYB* homolog, *AfimMYB114-like* is the only regulatory gene that is up-regulated in all three perianth parts (limb, tube, and utricle) in the S6 developmental stage. This indicates that the SG6 *R2R3-MYB* genes are good candidate transcription factors to activate early enzymes in the pathway, similar to the SG7 *R2R3-MYB* enzymes in *Arabidopsis*, and later as part of the MBW complex (Zhao et al., 2013). The bHLH homologs, *AfimTT8* and *AfimGL3-like* genes are up-regulated in the tube at S6, which is also the first part of the perianth to turn dark purple during development. Conversely, the WD40 homolog, *AfimTTG1* gene was found to be up-regulated in S9, but not S6. The correlated expression of the SG6 *R2R3-MYB* genes and the bHLH TFs in the absence of a WD40 partner suggests a simpler MB complex in early divergent species, when compared to a complete MBW complex in model core eudicots. Alternatively, a complex may not be needed until late (S9) developmental stages were anthocyanins are more actively produced. All of these are testable hypotheses for future functional analyses.

The WD40 transcription factor *TTG1* and all enzymes of the flavonoid pathway evaluated here are up-regulated in the S9 stage. *AfimCHS*, the critical starting factor of the flavonoid pathway, appears to be up-regulated in the tube and the limb at S9 flowers. All enzymes, except *AfimCHS*, are up-regulated in the utricle. Such contrasting level of regulation could be correlated to the predominantly yellowish background of the utricle, and the restriction of dark purple to brown color to small spots associated with nectar production.

All Major Flavonoid and Anthocyanin Pathway Enzymes Are Present in Most Aristolochiaceae Species

The glycosylated forms of anthocyanidins, namely cyanidin, delphinidin, and pelargonidin are the most common anthocyanidins in flowering plants. Here, the absence/presence of the biosynthetic pathway enzymes for the formation of these three anthocyanins was evaluated for selected Aristolochiaceae species. Our *In silico* approach consisted on using predicted peptides from mixed transcriptomes resulting from total RNA extracted from leaves, flowers and fruits (if available) and mapping the complete routes in KEGG. These analyses can serve as a proxy to get preliminary observations on the conservation of complete pathways in non-model species. However, as the data comes from transcriptomic raw data, these are putative enzymes active at particular plant organs in specific developmental stages (see materials and methods).

Our results show that the enzyme Flavonoid 3′5′-hydroxylase is lacking in all examined species, which suggests that delphinidin is not produced. Delphinidin is better known for producing blue hues. For example, in *Hydrangea* flowers delphinidin-3-glucoside can form blue complexes by interacting with metal ions, especially Al^{3+} and Fe^{3+} (Yoshida et al., 2003; Schreiber et al., 2010), and blue is not normally part of the floral display in Aristolochiaceae.

All other major flavonoid pathway enzymes were found in most examined species, except in *Aristolochia arborea* and *A. clematitis*. KEGG results show that cyanidin and pelargonidin, two anthocyanidins mostly responsible for red and red-like colors (Chung et al., 2016), are synthesized in all species studied,

except in *A. arborea* and *A. clematitis*. Red and dark red colors are common in the perianth of many *Aristolochia* species; nevertheless, there was no peptide predicted that matched an anthocyanidin synthase (ANS) in *A. clematitis* or in *A. arborea.* In addition, no match was found for the bifunctional enzyme Dihydroflavonol 4-reductase/flavanone 4-reductase (DFR) in *A. arborea*. Given that the plant tissue used for the transcriptomes contained a mix of flowers and leaves in all the species, it makes sense that we were able to map all major enzymes in the yellow-flowered *A. clematitis*, as these enzymes are present and active in leaves even if anthocyanins are not being produced in the flowers. However, the absence of the ANS and DFR in *A. arborea* and ANS in *A. clematitis* from the predicted peptides may reflect either a biological process, such as the impact of environmental factors to which plants were exposed when tissue samples were taken, or an artifact resulting from low match in peptide sequence prediction. To support the former scenario, it is important to note that anthocyanin synthesis in fruits is affected by environmental conditions, such as light, temperature and pH (Jaakola, 2013). Cauliflory in *A. arborea* results in the production of flowers at the ground level of shaded tropical forests; it has been reported that flavonoid/anthocyanin pathway structural gene expression (like CHS and F3H) can be lowered by shading (He et al., 2010). To support the latter assumption, it is important to highlight that the UFGT enzyme was not found in any of the evaluated species by KEGG. This may be due to high divergence between the predicted peptide for these non-model Aristolochiaceae species when compared to the references in the KEGG database. We were able to isolate this enzyme from directed searches in our own *A. fimbriata* RNA-seq data, suggesting that it is an artifact of the *In silico* translation for some proteins.

CONCLUSION

Taken together, the phylogenetic reconstruction of the evolution of *R2R3-MYB* genes combined with the targeted expression analyses and the mapping of biosynthetic routes *in silico* shows that the flavonoid and early enzymes of the anthocyanin biosynthetic pathways are present and active in the Aristolochiaceae family. Importantly, single copy upstream regulators likely perform the same roles as those found duplicated in core eudicots. Moreover, when duplications were found, like in *Asarum* and *Saruma*, divergent expression of the copies is detected suggesting functional diversification after duplication. The fact that SG6 *R2R3-MYB* genes are found in leaves and sometimes even in yellow floral parts (like is the case for *SaheMYB114-like_a*) suggests more pleiotropic roles for these homologs when compared to their equivalents in rosids and asterids. Our results show that the SG6 *R2R3-MYB* single copy genes controlling the flavonoid biosynthetic pathways are simplified in the Aristolochiaceae and that a more complex regulation has evolved later in core eudicots with a larger copy number, such as in the Brassicales. Our data also shows that other members of the MBW complex are present as single copy genes and are active in Aristolochiaceae. The scenario that we present for SG6 *R2R3-MYB* genes poses the Aristolochiaceae as a suitable family to study complex color floral patterns with a relatively simple genetic bases with single copy regulators and a complete active pathway.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The datasets generated for this study can be found in the NCBI GenBank accessions MW125647-MW125662.

AUTHOR CONTRIBUTIONS

SM-G, HS-B, FG, and NP-M planned and designed the research, conducted fieldwork, and performed the experiments. SM-G, HS-B, NP-M, and JA executed the bioinformatics analysis. SM-G, HS-B, JA, FG, and NP-M analyzed the data, and wrote and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.633227/full#supplementary-material

Supplementary Figure 1 | Comprehensive Maximum likelihood analysis of the SG6 *R2R3-MYB* genes with an expanded outgroup including SG4, 5, 7, and 15. Outgroup used here is *AtMYB3*.

Supplementary Figure 2 | Protein sequence alignment of SG6 R2R3-MYB selected proteins showing the diagnostic *R2R3-MYB* motif for subgroup 6 as reported by Stracke et al., 2001 boxed.

Supplementary Figure 3 | Developmental stages sampled for RT-PCR of **(A)** *Saruma henryi*, **(B)** *Asarum canadense*, **(C)** *Aristolochia fimbriata*, **(D)** *A. manshuriensis*. *l*, limb; *p*, petal; *s*, sepal; *t*, tube; *u*, utricle.

Supplementary Figure 4 | Image J quantitative expression analysis based on the original electrophoresis images obtained by RT-PCR with an unprocessed background.

Supplementary Figure 5 | Maximum likelihood analysis of the *EGL1/GL3* bHLH genes across angiosperms. Yellow stars indicate large-scale duplication events. Color clades follow the conventions in the top left. The *Aristolochia fimbriata* homolog is pointed with a red arrow.

Supplementary Figure 6 | Maximum likelihood analysis of the *TT8* bHLH genes across angiosperms. Color clades follow the conventions in the top left. The *Aristolochia fimbriata* homolog is pointed with a red arrow.

Supplementary Figure 7 | Maximum likelihood analysis of the *TTG1* WD40 genes across angiosperms. Color clades follow the conventions in the top left. The *Aristolochia fimbriata* homolog is pointed with a red arrow.

Supplementary Table 1 | Names, abbreviations, codes, and original databases for all sequences included in the SG6 R2R3 MYB phylogenetic analyses.

Supplementary Table 2 | Name, sequence, and melting temperature of the primers used during RT-PCR analyses.

Supplementary Table 3 | *De novo* assembly of the floral transcriptomes of *Aristolochia fimbriata* at two different developmental stages.

Supplementary Table 4 | Expression of the candidate genes included in this study. Values correspond to the TPMs.

Supplementary Table 5 | Names, abbreviations, codes, and original databases for all sequences included in the bHLH phylogenetic analyses.

Supplementary Table 6 | Names, abbreviations, codes, and original databases for all sequences included in the WD40 phylogenetic analyses.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Bernardello, G., Anderson, G. J., Lopez, S. P., Cleland, M. A., Stuessy, T. F., and Crawford, D. J. (1999). Reproductive biology of *Lactoris fernandeziana* (Lactoridaceae). *Am. J. Bot.* 86, 829–840. doi: 10.2307/2656704

Bliss, B. J., Wanke, S., Barakat, A., Ayyampalayam, S., Wickett, N., Kerr Wall, P., et al. (2013). Characterization of the basal angiosperm *Aristolochia fimbriata*: a potential experimental system for genetic studies. *BMC Plant Biol.* 13:13. doi: 10.1186/1471-2229-13-13

Borevitz, J. O., Xia, Y., Blount, J., Dixon, R. A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 12, 2383–2393.

Bradshaw, H. D., Wilbert, S. M., Otto, K. G., and Schemske, D. W. (1995). Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (Mimulus). *Nature* 376, 762–765. doi: 10.1038/376762a0

Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* 34, 525–528. doi: 10.1038/nbt0816-888d

Calderaro, A., Barreca, D., Bellocco, E., Smeriglio, A., Trombetta, D., and Laganà, G. (2020). "Colored phytonutrients: role and applications in the functional foods of anthocyanins," in *Phytonutrients in Food*, eds D. Barreca, H. Khan, I. Suntar, and S. M. Nabavi (Sawston: Woodhead Publishing), 177–195.

Chatham, L. A., Paulsmeyer, M., and Juvik, J. A. (2019). Prospects for economical natural colorants: insights from maize. *Theor. Appl. Genet.* 132, 2927–2946. doi: 10.1007/s00122-019-03414-0

Chung, S. W., Yu, D. J., and Lee, H. J. (2016). Changes in anthocyanidin and anthocyanin pigments in highbush blueberry (*Vaccinium corymbosum* cv. Bluecrop) fruits during ripening. *Hortic. Environ. Biotechnol.* 57, 424–430. doi: 10.1007/s13580-016-0107-8

Clement, J. S., Mabry, T. J., Wyler, H., and Dreiding, A. S. (1994). "Chemical review and evolutionary significance of the betalains," in *Caryophyllales, evolution and Systematics*, eds H.-D. Behnke and T. J. Mabry (Berlin: Springer), 247–261.

Clifford, M. N. (2000). Anthocyanins–nature, occurrence and dietary burden. *J. Sci. Food Agric.* 80, 1063–1072. doi: 10.1002/(SICI)1097-0010(20000515)80:7

Davies, K. M., Albert, N. W., and Schwinn, K. E. (2012). From landing lights to mimicry: the molecular regulation of flower coloration and mechanisms for pigmentation patterning. *Funct. Plant Biol.* 39, 619–638. doi: 10.1071/FP12195

Dietrich, P., Sanders, D., and Hedrich, R. (2001). The role of ion channels in light-dependent stomatal opening. *J. Exp. Bot.* 52, 1959–1967. doi: 10.1093/jexbot/52.363.1959

Ding, B., Patterson, E. L., Holalu, S. V., Li, J., Johnson, G. A., Stanley, L. E., et al. (2020). Two MYB proteins in a self-organizing activator-inhibitor system produce spotted pigmentation patterns. *Curr. Biol.* 30, 802–814. doi: 10.1016/j.cub.2019.12.067

Du, H., Liang, Z., Zhao, S., Nan, M. G., Tran, L. S. P., Lu, K., et al. (2015). The evolutionary history of R2R3-MYB proteins across 50 eukaryotes: new insights into subfamily classification and expansion. *Sci. Rep.* 5:11037. doi: 10.1038/srep11037

Dubos, C., Le Gourrierec, J., Baudry, A., Huep, G., Lanet, E., Debeaujon, I., et al. (2008). MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. *Plant J.* 55, 940–953. doi: 10.1111/j.1365-313X.2008.03564.x

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010). MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.* 15, 573–581. doi: 10.1016/j.tplants.2010.06.005

Fang, Z., Hou, Z., Wang, S., Liu, Z., Wei, S., Zhang, Y., et al. (2019). Transcriptome analysis reveals the accumulation mechanism of anthocyanins in buckwheat (*Fagopyrum esculentum* Moench) cotyledons and flowers. *Int. J. Mol. Sci.* 20:1493. doi: 10.3390/ijms20061493

Feng, K., Xu, Z. S., Que, F., Liu, J. X., Wang, F., and Xiong, A. S. (2018). An R2R3-MYB transcription factor, OjMYB1, functions in anthocyanin biosynthesis in *Oenanthe javanica*. *Planta* 247, 301–315. doi: 10.1007/s00425-017-2783-8

Frey, F. M., Dunton, J., and Garland, K. (2011). Floral color variation and associations with fitness-related traits in *Malva moschata* (Malvaceae). *Plant Species Biol.* 26, 235–243. doi: 10.1111/j.1442-1984.2011.00325.x

Gates, D. J., Olson, B. J., Clemente, T. E., and Smith, S. D. (2018). A novel R3 MYB transcriptional repressor associated with the loss of floral pigmentation in Iochroma. *New Phytol.* 217, 1346–1356. doi: 10.1111/nph.14830

González, A., Zhao, M., Leavitt, J. M., and Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant J.* 53, 814–827. doi: 10.1111/j.1365-313X.2007.03373.x

González, F., and Rudall, P. J. (2001). The questionable affinities of Lactoris: evidence from branching pattern, inflorescence morphology, and stipule development. *Am. J. Bot.* 88, 2143–2150.

González, F., and Stevenson, D. W. (2000). Perianth development and systematics of *Aristolochia*. *Flora* 195, 370–391. doi: 10.1016/S0367-2530(17)30995-7

Guo, X., Liu, J., Hao, G., Zhang, L., Mao, K., Wang, X., et al. (2017). Plastome phylogeny and early diversification of Brassicaceae. *BMC Genomics* 18:176. doi: 10.1186/s12864-017-3555-3

He, F., Mu, L., Yan, G. L., Liang, N. N., Pan, Q. H., Wang, J., et al. (2010). Biosynthesis of anthocyanins and their regulation in colored grapes. *Molecules* 15, 9057–9091. doi: 10.3390/molecules15129057

Higginson, T., Li, S. F., and Parish, R. W. (2003). AtMYB103 regulates tapetum and trichome development in *Arabidopsis thaliana*. *Plant J.* 35, 177–192. doi: 10.1046/j.1365-313X.2003.01791.x

Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q., and Vinh, L. S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Mol. Biol. Evol.* 35, 518–522. doi: 10.1093/molbev/msx281

Holton, T. A., and Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7:1071. doi: 10.1105/tpc.7.7.1071

Hsu, C. C., Chen, Y. Y., Tsai, W. C., Chen, W. H., and Chen, H. H. (2015). Three R2R3-MYB transcription factors regulate distinct floral pigmentation patterning in Phalaenopsis spp. *Plant Physiol.* 168, 175–191.

Jaakola, L. (2013). New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends Plant Sci.* 18, 477–483. doi: 10.1016/j.tplants.2013.06.003

Jackman, R. L., and Smith, J. L. (1996). "Anthocyanins and betalains," in *Natural Food Colorants*, eds G. A. F. Hendry and J. D. Houghton (Boston, MA: Springer), 244–309.

Jin, J., Tian, F., Yang, D. C., Meng, Y. Q., Kong, L., Luo, J., et al. (2016). PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.* 45, D1040–D1045. doi: 10.1007/978-1-4615-2155-6_8

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K., von Haeseler, A., and Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* 14, 587–589. doi: 10.1038/nmeth.4285

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30.

Katoh, K., Rozewicki, J., and Yamada, K. D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* 20, 1160–1166. doi: 10.1093/bib/bbx108

Khomtchouk, B. B., Hennessy, J. R., and Wahlestedt, C. (2017). Shinyheatmap: ultra-fast low memory heatmap web interface for big data genomics. *PLoS One* 12:e0176334. doi: 10.1371/journal.pone.0176334

Kidd, P. S., Llugany, M., Poschenrieder, C. H., Gunse, B., and Barcelo, J. (2001). The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays* L.). *J. Exp. Bot.* 52, 1339–1352. doi: 10.1093/jexbot/52.359.1339

Klempnauer, K. H., Gonda, T. J., and Michael Bishop, J. (1982). Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. *Cell* 31, 453–463. doi: 10.1016/0092-8674(82)90138-6

Kong, J. M., Chia, L. S., Goh, N. K., Chia, T. F., and Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry* 64, 923–933. doi: 10.1016/S0031-9422(03)00438-2

Kranz, H. D., Denekamp, M., Greco, R., Jin, H., Leyva, A., Meissner, R. C., et al. (1998). Towards functional characterization of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant J.* 16, 263–276. doi: 10.1046/j.1365-313x.1998.00278.x

Leebens-Mack, J. H., Barker, M. S., Carpenter, E. J., Deyholos, M. K., Gitzendanner, M. A., Graham, S. W., et al. (2019). One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574, 679–685. doi: 10.1038/s41586-019-1693-2

Li, B.-J., Zheng, B.-Q., Wang, J.-Y., Tsai, W.-C., Lu, H.-C., Zou, L.-H., et al. (2020). New insight into the molecular mechanism of colour differentiation among floral segments in orchids. *Commun. Biol.* 3:89. doi: 10.1038/s42003-020-0821-8

Li, J., Ren, L., Gao, Z., Jiang, M., Liu, Y., Zhou, L., et al. (2017). Combined transcriptomic and proteomic analysis constructs a new model for light-induced anthocyanin biosynthesis in eggplant (*Solanum melongena* L.). *Plant Cell Environ.* 40, 3069–3087. doi: 10.1111/pce.13074

Li, S. (2014). Transcriptional control of flavonoid biosynthesis: fine-tuning of the MYB-bHLH-WD40 (MBW) complex. *Plant Signal. Behav.* 9:e27522. doi: 10.4161/psb.27522

Li, W., Liu, Y., Zeng, S., Xiao, G., Wang, G., Wang, Y., et al. (2015). Gene expression profiling of development and anthocyanin accumulation in kiwifruit (*Actinidia chinensis*) based on transcriptome sequencing. *PLoS One* 10:e0136439. doi: 10.1371/journal.pone.0136439

Lin, R.-C., and Rausher, M. D. (2020). R2R3-MYB genes control petal pigmentation patterning in *Clarkia gracilis* ssp. sonomensis (Onagraceae). *New Phyt.* 229:2. doi: 10.1111/nph.16908

Lin-Wang, K., Bolitho, K., Grafton, K., Kortstee, A., Karunairetnam, S., McGhie, T. K., et al. (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC Plant Biol.* 10:50. doi: 10.1186/1471-2229-10-50

Liu, Y., Lin-Wang, K., Espley, R. V., Wang, L., Yang, H., Yu, B., et al. (2016). Functional diversification of the potato R2R3 MYB anthocyanin activators AN1, MYBA1, and MYB113 and their interaction with basic helix-loop-helix cofactors. *J. Exp. Bot.* 67, 2159–2176. doi: 10.1093/jxb/erw014

Lowry, D. B., Sheng, C. C., Lasky, J. R., and Willis, J. H. (2012). Five anthocyanin polymorphisms are associated with an R2r3-Myb cluster in *Mimulus guttatus* (Phrymaceae). *Am. J. Bot.* 99, 82–91. doi: 10.3732/ajb.1100285

Matsui, K., and Walker, A. R. (2019). Biosynthesis and regulation of flavonoids in buckwheat. *Breed. Sci.* 70, 74–84. doi: 10.1270/jsbbs.19041

Morita, Y., Saitoh, M., Hoshino, A., Nitasaka, E., and Iida, S. (2006). Isolation of cDNAs for R2R3 MYB, bHLH and WDR transcriptional regulators and identification of c and ca mutations conferring white flowers in the Japanese morning glory. *Plant Cell Physiol.* 47, 457–470. doi: 10.1093/pcp/pcj012

Musselman, L. J., and Visser, J. H. (1989). Taxonomy and natural history of Hydnora (Hydnoraceae). *Aliso* 12, 317–326.

Narbona, E., Buide, M. L., Casimiro-Soriguer, I., and del Valle, J. C. (2014). Polimorfismos de color floral: causas e implicaciones evolutivas. *Ecosistemas* 23, 36–47. doi: 10.7818/ECOS.2014.23-3.06

Noda, K. I., Glover, B. J., Linstead, P., and Martin, C. (1994). Flower color intensity depends on specialized cell shape controlled by a Myb-related transcription factor. *Nature* 369, 661–664.

Pabón-Mora, N., Madrigal, Y., Alzate, J. F., Ambrose, B. A., Ferrándiz, C., Wanke, S., et al. (2020). Class II TCP gene evolution in perianth-bearing Piperales and their contribution to the bilateral calyx in *Aristolochia*. *New Phytol.* 228, 752–769. doi: 10.1111/nph.16719

Pabón-Mora, N., Suárez-Baron, H., Ambrose, B. A., and González, F. (2015). Flower development and perianth identity candidate genes in the basal angiosperm *Aristolochia fimbriata* (Piperales: aristolochiaceae). *Front. Plant Sci.* 6:1095. doi: 10.3389/fpls.2015.01095

Park, J. S., Kim, J. B., Cho, K. J., Cheon, C. I., Sung, M. K., Choung, M. G., et al. (2008). Arabidopsis R2R3-MYB transcription factor AtMYB60 functions as a transcriptional repressor of anthocyanin biosynthesis in lettuce (*Lactuca sativa*). *Plant Cell. Rep.* 27, 985–994. doi: 10.1007/s00299-008-0521-1

Pérez-Díaz, J. R., Pérez-Díaz, J., Madrid-Espinoza, J., González-Villanueva, E., Moreno, Y., and Ruiz-Lara, S. (2016). New member of the R2R3-MYB transcription factors family in grapevine suppresses the anthocyanin accumulation in the flowers of transgenic tobacco. *Plant Mol. Biol.* 90, 63–76. doi: 10.1007/s11103-015-0394-y

Petroni, K., and Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Sci.* 181, 219–229. doi: 10.1016/j.plantsci.2011.05.009

Qi, Y., Gu, C., Wang, X., Gao, S., Li, C., Ma, C., et al. (2020). Identification of the Eutrema salsugineum EsMYB90 gene important for anthocyanin biosynthesis. *BMC Plant Biol.* 20:1–15. doi: 10.21203/rs.2.18301/v2

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., et al. (1999). Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. *Plant Cell* 11, 1433–1444. doi: 10.1105/tpc.11.8.1433

Romero, I., Fuertes, A., Benito, M. J., Malpica, J. M., Leyva, A., and Paz-Ares, J. (1998). More than 80R2R3-MYB regulatory genes in the genome of *Arabidopsis thaliana*. *Plant J.* 14, 273–284. doi: 10.1046/j.1365-313x.1998.00113.x

Sarma, A. D., and Sharma, R. (1999). Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. *Phytochemistry* 52, 1313–1318. doi: 10.1016/S0031-9422(99)00427-6

Schreiber, H. D., Swink, A. M., and Godsey, T. D. (2010). The chemical mechanism for Al3+ complexing with delphinidin: a model for the bluing of hydrangea sepals. *J. Inorg. Biochem.* 104, 732–739. doi: 10.1016/j.jinorgbio.2010.03.006

Schwinn, K., Venail, J., Shang, Y. J., Mackay, S., Alm, V., Butelli, E., et al. (2006). A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus Antirrhinum. *Plant Cell* 18, 831–851. doi: 10.1105/tpc.105.039255

Schwinn, K. E., Ngo, H., Kenel, F., Brummell, D. A., Albert, N. W., McCallum, J. A., et al. (2016). The onion (*Allium cepa* L.) R2R3-MYB gene MYB1 regulates anthocyanin biosynthesis. *Front. Plant Sci.* 7:1865. doi: 10.3389/fpls.2016.01865

Shaiju, P. N., and Omanakumari, N. (2010). Floral morphology and systematics of the genus Thottea Rottb. (Aristolochiaceae) from the Western Ghats, India. *Plant Syst. Evol.* 288, 213–225. doi: 10.1007/s00606-010-0326-x

Shang, Y. J., Venail, J., Mackay, S., Bailey, P. C., Schwinn, K. E., Jameson, P. E., et al. (2011). The molecular basis for venation patterning of pigmentation and its effect on pollinator attraction in flowers of Antirrhinum. *New Phytol.* 189, 602–615. doi: 10.1111/j.1469-8137.2010.03498.x

Stracke, R., Werber, M., and Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol.* 4, 447–456. doi: 10.1016/S1369-5266(00)00199-0

Sun, W., Ma, Z., Chen, H., and Liu, M. (2019). MYB gene family in potato (*Solanum tuberosum* L.): genome-wide identification of hormone-responsive reveals their potential functions in growth and development. *Int. J. Mol. Sci.* 20:4847. doi: 10.3390/ijms20194847

Tanaka, Y., Sasaki, N., and Ohmiya, A. (2008). Plant pigments for coloration: anthocyanins, betalains and carotenoids. *Plant J.* 54, 733–749. doi: 10.1111/j.1365-313X.2008.03447.x

Tominaga, R., Iwata, M., Sano, R., Inoue, K., Okada, K., and Wada, T. (2008). Arabidopsis CAPRICE-LIKE MYB 3 (CPL3) controls endoreduplication and flowering development in addition to trichome and root hair formation. *Development* 135, 1335–1345. doi: 10.1242/dev.017947

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., and Minh, B.-Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for máximum likelihood analysis. *Nuclie Acids Res.* 44, 232–235. doi: 10.1093/nar/gkw256

Wagner, S. T., Hesse, L., Isnard, S., Samain, M. S., Bolin, J., Maass, E., et al. (2014). Major trends in stem anatomy and growth forms in the perianth-bearing Piperales, with special focus on Aristolochia. *Ann. Bot.* 113, 1139–1154. doi: 10.1093/aob/mcu044

Wagner, S. T., Isnard, S., Rowe, N. P., Samain, M. S., Neinhuis, C., and Wanke, S. (2012). Escaping the lianoid habit: evolution of shrub-like growth forms in *Aristolochia subgenus* Isotrema (Aristolochiaceae). *Am. J. Bot.* 99, 1609–1629. doi: 10.3732/ajb.1200244

Wang, Z., Tang, J., Hu, R., Wu, P., Hou, X. L., Song, X. M., et al. (2015). Genome-wide analysis of the R2R3-MYB transcription factor genes in Chinese cabbage (*Brassica rapa* ssp. pekinensis) reveals their stress and hormone responsive patterns. *BMC Genomics* 16:17. doi: 10.1186/s12864-015-1216-y

Wanke, S., González, F., and Neinhuis, C. (2006). Systematics of pipevines: combining morphological and fast-evolving molecular characters to investigate the relationships within subfamily Aristolochioideae (Aristolochiaceae). *Int. J. Plant Sci.* 167, 1215–1227. doi: 10.1086/508024

Wilkins, O., Nahal, H., Foong, J., Provart, N. J., and Campbell, M. M. (2009). Expansion and diversification of the Populus R2R3-MYB family of transcription factors. *Plant Physiol.* 149, 981–993. doi: 10.1104/pp.108.132795

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126, 485–493. doi: 10.1104/pp.126.2.485

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.* 5, 218–223. doi: 10.1016/S1369-5266(02)00256-X

Wittall, J. B., Voelckel, C., Kliebenstein, D. J., and Hodges, S. A. (2006). Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in Aquilegia. *Mol. Ecol.* 15, 4645–4657. doi: 10.1111/j.1365-294X.2006.03114.x

Wu, C. A., Streisfeld, M. A., Nutter, L. I., and Cross, K. A. (2013). The genetic basis of a rare flower color polymorphism in Mimulus lewisii provides insight into the repeatability of evolution. *PLoS One* 8:e81173. doi: 10.1371/journal.pone.0081173

Yamagishi, M. (2016). A novel R2R3-MYB transcription factor regulates light-mediated floral and vegetative anthocyanin pigmentation patterns in Lilium regale. *Mol. Breed.* 36:3. doi: 10.1007/s11032-015-0426-y

Yao, G., Ming, M., Allan, A. C., Gu, C., Li, L., Wu, X., et al. (2017). Map-based cloning of the pear gene MYB 114 identifies an interaction with other transcription factors to coordinately regulate fruit anthocyanin biosynthesis. *Plant J.* 92, 437–451. doi: 10.1111/tpj.13666

Yoshida, K., Toyama-Kato, Y., Kameda, K., and Kondo, T. (2003). Sepal color variation of Hydrangea macrophylla and vacuolar pH measured with a proton-selective microelectrode. *Plant Cell Physiol.* 44, 262–268.

Zhang, B., and Schrader, A. (2017). TRANSPARENT TESTA GLABRA 1-dependent regulation of flavonoid biosynthesis. *Plants* 6:65. doi: 10.3390/plants6040065

Zhang, L., Chen, F., Zhang, X., Li, Z., Zhao, Y., Lohaus, R., et al. (2020). The water lily genome and the early evolution of flowering plants. *Nature* 577, 79–84. doi: 10.1038/s41586-019-1852-5

Zhao, L., Gao, L., Wang, H., Chen, X., Wang, Y., Yang, H., et al. (2013). The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Funct. Inter. Genomic* 13, 75–98. doi: 10.1007/s10142-012-0301-4

Zufall, R. A., and Rausher, M. D. (2003). The genetic basis of a flower color polymorphism in the common morning glory (*Ipomoea purpurea*). *J. Hered.* 94, 442–448. doi: 10.1093/jhered/esg098

Zuluaga, D. L., Gonzali, S., Loreti, E., Pucciariello, C., Degl'Innocenti, E., Guidi, L., et al. (2008). Arabidopsis thaliana MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. *Funct. Plant Biol.* 35, 606–618. doi: 10.1071/FP08021

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Floral UV Features of Plant Species From a Neotropical Savanna

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Despite the wide interest in flower colours, only after the end of the nineteenth-century studies started to comprise floral UV reflection, which is invisible to humans but visible to the major groups of pollinators. Many flowers and inflorescences display colour patterns, an important signal for pollinators, promoted by the presence of at least two different colours within flowers or inflorescences, including colours in the UV waveband. For Neotropical savanna plant species, we characterised floral UV features using UV-photography and reflectance measurements. We tested (i) whether floral UV features were constrained by their shared ancestry, (ii) whether floral UV features were associated with pollinators, and (iii) whether floral UV features were associated with floral traits mediating these interactions, including floral resource, type of attraction unit and presence/absence of non-UV colour patterns. Of 80 plant species, ca. 70% were UV-patternless, most of them UV-absorbing. Approximately 30% presented one of three types of UV-patterns: bullseye, contrasting corolla markings oriented toward floral resources or contrasting reproductive structures, which were all considered as floral guides. Floral UV features were phylogenetically constrained and were associated with pollinators, floral resources and attraction unit, but not with non-UV colour patterns. UV-patternless flowers were associated with most of the pollination systems, while UV-patterned flowers were mainly associated with bee-pollination. UV-absorbing flowers comprised the only category with hawkmoth- and butterfly-pollinated flowers, and a high percentage of hummingbird-pollinated species. Nocturnal pollinated species were also commonly UV-absorbing, except for one UV-reflecting bat-pollinated species and one beetle-pollinated species with UV-reflecting stigmas. All types of floral UV features were associated with nectar; however, flowers with contrasting reproductive structures were mainly associated with pollen. There was an association between UV-absorbing species and the presence of inflorescences and intermediate attraction units. Our results evince that phylogenetic relatedness can constraint floral UV features' diversification, but combinations of evolutionary and ecological processes may be expected in this scenario.

Keywords: biodiversity, floral guides, floral resource, flower colour, pollination systems, phylogeny, ultraviolet reflectance, UV-pattern

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INTRODUCTION

Floral colour has always called humankind's attention, and throughout time it was explored in several studies including genetic, ecological, and evolutionary approaches. However, until the end of the nineteenth century, these studies did not comprise the reflection of UV light, which was just recorded in flowers in 1891 by Knuth (1891). Ever since floral UV reflection has been attracting scientists' interest as UV light is invisible to humans but visible to the major groups of pollinators. Indeed, floral UV-colouring can be perceived by many pollinators, such as bees (Chittka and Briscoe, 2001; Cronin et al., 2014), hummingbirds (Goldsmith and Goldsmith, 1979; Ödeen and Håstad, 2010), flies (Chittka and Briscoe, 2001; Cronin et al., 2014; Lunau, 2014), butterflies (Chittka and Briscoe, 2001; Cronin et al., 2014), beetles (Chittka and Briscoe, 2001), hawkmoths (White et al., 1994; Cronin et al., 2014), and bats (Müller et al., 2009), since all of these pollinator groups present photoreceptors that are sensitive to UV wavelengths. It is important to highlight that from a pollinator's perspective, the UV wavelengths are only one of the wavebands perceived by animals that have UV photoreceptors (Kevan, 1979; Cronin et al., 2014). It acts together with the information from other photoreceptors that may perceive longer wavelengths, encompassing human-visible blue, green and red wavebands to create a colour (Chittka et al., 1994).

Floral colour diversity encompasses flowers that can be uniform in colour (non-patterned) and flowers that display colour patterns (patterned) (Koski, 2020). Floral colour patterns are formed by contrasting portions on the flowers, in the perianth or reproductive structures, which can be perceived by pollinators' sensory systems (Chittka and Thomson, 2001). Floral UV-patterns, in the same way as human-visible patterns, are created by the local accumulation of pigments that can be UV-absorbing, such as flavonoids, including flavonols, chalcones and most anthocyanins (Thompson et al., 1972; Harborne, 1981; Kevan et al., 1996 and references therein; Schlangen et al., 2009), or UV-reflecting, such as carotenoids and some anthocyanins (Kevan et al., 1996 and references therein; Schlangen et al., 2009). Plant species that are more closely related in terms of phylogeny could show similar UV floral patterns (as shown for some clades of Rosaceae by Koski, 2020) since pigment biosynthesis and allocation can be genetically determined and regulated (Grotewold, 2006). Indeed, Camargo et al. (2019) showed that the amount of excitation caused by bee-pollinated flowers and hummingbird-pollinated flowers in their respective pollinator's UV-photoreceptor was phylogenetically structured. However, still little is known about the evolutionary history of UV-patterns (Koski, 2020).

Although insect vision is limited to detect flower colour patterns at long-distance ranges (Hempel de Ibarra et al., 2015 and references therein), these patterns set important cues to discriminate among flowers at close range, to guide landing onto the flowers (Lunau, 1992; Hempel de Ibarra et al., 2015), and to locate resources within the flowers (Daumer, 1956; Dinkel and Lunau, 2001; Leonard and Papaj, 2011), thus, mediating plant-pollinator interactions (Medel et al., 2003; Koski and Ashman, 2014). Additionally, colour patterns can influence pollinator constancy and preferences (Hill et al., 1997; Bradshaw and Schemske, 2003; Horth et al., 2014), affecting the success of pollination (Johnson and Dafni, 1998; Goodale et al., 2014; Koski and Ashman, 2015). Furthermore, changes in UV features in flowers can even be associated with pollinator shifts (Bradshaw and Schemske, 2003; Sheehan et al., 2016; Martínez-Harms et al., 2020). Thus, it would be expected that the distribution of the types of floral UV features is shaped by pollinator preferences and behaviour through pollinator-mediated selection, as other floral traits (Jogesh et al., 2017).

Additionally, UV floral patterns can act as guides, used by pollinators to locate floral resources (Lunau, 1992; Koski and Ashman, 2014; Lunau et al., 2020). However, to our knowledge, possible associations between types of UV floral patterns and the types of floral resources presented by plant species; has not yet been tested. Nevertheless, pollinators might use flowers or inflorescences to make foraging decisions (Burdon et al., 2020), so that in some cases flowers act as attraction units and in other, inflorescences do (Ramirez et al., 1990). Thus, the pattern displayed by individual flower creates, together, a unique pattern that represents the inflorescence as a unit, such as a bullseye pattern, commonly displayed by Compositae inflorescences (e.g., Milne and Milne, 1958; Abrahamson and McCrea, 1977; Moyers et al., 2017). Consequently, there could be an association of the type of UV floral patterns presented by plants and their type of attraction unit.

More recently, the number of studies focused on understanding the distribution of floral UV patterns among flowering plants and on understanding the implications of such floral patterns to plant-pollinator interactions have increased substantially. However, there is still a wide range of questions to be answered regarding floral UV patterns. Here, we used plant species from a Neotropical savanna to characterise floral UV features and tested (i) whether floral UV features were constrained by their shared ancestry, (ii) whether floral UV features were related to pollinators, and (iii) whether floral UV features were associated with floral traits mediating these interactions, including floral resource, type of attraction unit and the presence/absence of non-UV colour patterns.

MATERIALS AND METHODS

Study Sites

This study was conducted in natural populations of savanna physiognomies from "cerrado" vegetation, located in Botucatu (22°54'45" S, 48°30'13" W), Águas de Santa Bárbara ("Santa Bárbara Ecological Station," 22°46'–22°41'S, 49°16'–49°10'W) municipalities, in São Paulo state, and São Roque de Minas municipality ("Serra da Canastra National Park," 20°14'01" S, 46°26'40" W), in Minas Gerais state, Brazil. Sisgen authorisation #A90A83C and ICMBio/MMA/SISBIO authorisation #70131-1 for collection of biological samples. The "cerrado," is a highly diverse Neotropical savanna vegetation (Pfadenhauer and Klötzli, 2020) in which flowering species are mainly pollinated by bees, but also by other vectors, such as hummingbirds, bats, flies, butterflies, beetles and moths

(Gottsberger and Silberbauer-Gottsberger, 2006). To ensure that the proportion of pollination systems in our assemblage was representative of "cerrado" communities in general, we compared the observed ratios with those described by Gottsberger and Silberbauer-Gottsberger (2006) and Tunes et al. (2017) for other "cerrado" communities. For that, we used the Kruskall-Wallis test, after checking non-normality of the data. We found that the proportions of pollination systems in our study were similar to those recorded by Gottsberger and Silberbauer-Gottsberger (2006) and Tunes et al. (2017) ($\chi^2 = 0.47043$, $df = 2$, $p = 0.7904$; see the specific proportions in **Supplementary Table 1**), evincing the representativeness of our dataset.

Assessment of Floral UV Features

We performed various expeditions to survey the UV features from flowers of savanna species. We conducted the field study throughout the years of 2019 to 2021, to capture the blooming period of a large number of plant species. To access the UV features, we used UV-photography, which allows us to see if and which UV patterns were present in flowers and inflorescences. We also validated the observed patterns with reflectance data of different floral parts. Based on these complementary data, UV-photographs, and reflectance data, we described floral UV features. Then, we classified the observed features into categories.

We photographed the UV reflectance of one to three flowers and inflorescences of 80 plant species (**Supplementary Table 2**) in studio conditions. For that, we used a hand-held UV light source, which emits light from 315 to 405 nm, with a peak at 365 nm, to illuminate the flowers. We excluded all human-visible light by using a camera with a modified sensor that only captures UV light from 340 to 400 nm, which corresponds exclusively to the UV portion of the light spectrum. In addition, this range corresponds to the spectral sensitivity of the UV-photoreceptors of a large variety of Hymenopteran pollinators (Chittka, 1992; Peitsch et al., 1992; Skorupski et al., 2007; Skorupski and Chittka, 2010), bird pollinators (Herrera et al., 2008; Ödeen and Håstad, 2010), and bat pollinators (Müller et al., 2009).

To collect the reflectance data, we used a spectrophotometer (Ocean Optics Jaz-EL200 UV-VIS) which collects reflectance data from 200 to 890 nm including the UV and human-visible wavelengths. We considered UV-reflecting when a given floral part reflects more than 5% between 300 and 400 nm, the UV-band. Although the mean reflectance of our data in the UV-band is around 3.5%, we opted by 5% to make our classification comparable with other community studies (e.g., Chittka et al., 1994; Camargo et al., 2019). As complementary information, we checked if the reflectance of a given floral structure presented marker points (Shrestha et al., 2013; Bukovac et al., 2017) located in the UV-band, that is, a stimulus promoted by rapid reflectance changes between 300 and 400 nm. We surveyed the marker points according to Camargo et al. (2019), using the "peakshape" function of the "pavo" package for R (Maia et al., 2019).

Floral UV Features' Phylogenetic Signal

We built a phylogenetic tree of the sampled plant species with PhyloMaker based on the "Phytophylo" megaphylogeny (Qian and Jin, 2016). We built the phylogenetic tree based on the third scenario, which creates polytomies by adding absent genera or species to their closest taxa. Then, we used δ (Borges et al., 2019) to verify the degree of phylogenetic signal between the presence of a given type of floral UV feature and the species' phylogeny. This method calculates node entropy through a linear adaptation of Shannon entropy and then applies a Bayesian inferential scheme to calculate δ-value to verify the degree of phylogenetic signal in categorical traits (Borges et al., 2019). Higher gamma values indicate higher degrees of phylogenetic signal in the analysed categorical traits (Borges et al., 2019).

Pollinators

Besides classifying the flower UV features, we also classified every plant species according to their pollinators, as described in the literature. When pollinators' information was not available, we determined the most probable pollen vector based on flower attributes according to Faegri and Van der Pijl (1979) and Rosas-Guerrero et al. (2014). See **Supplementary Table 2** for pollinator information for every plant species.

Floral Resources and Attraction Units

We classified every plant species according to their floral resources, as described in the literature. If a plant species presented more than one type of floral resource, it was included in all the corresponding categories, since we cannot discriminate "*a priori*" which of the resources, present in each plant species, could be associated with its floral UV feature. We classified every plant species regarding their attraction unit, that is, single flowers, intermediate or inflorescences, based on the classification proposed by Ramirez et al. (1990). See **Supplementary Table 2** for floral resource and attraction unit information for every plant species.

We classified every plant species according to the presence/absence of non-UV colour pattern according to the human colour vision, which is sensitive for wavelengths from 400 to 700 nm. The presence/absence of non-UV colour patterns was confirmed by the reflectance data collected as previously described. See **Supplementary Table 2** for the presence/absence of non-UV colour patterns in every plant species.

Statistical Analyses

We calculated phylogenetic signal (δ) based on the method proposed by Borges et al. (2019) for categorical variables. To evaluate if there was any association between the frequencies of each type of floral UV feature and pollinator group, between the frequencies of each type of floral UV feature and floral resource type, between the frequencies of each type of floral UV feature and attraction unit type, and between the frequencies of each type of floral UV feature and the presence/absence of non-UV colour patterns, we performed asymptotic generalised Pearson chi-squared tests with *post hoc* pairwise tests of independence. We carried out the statistical analyses in R v. 4.0.2 (R Core Team, 2020) with standard and additional packages: ape (Paradis and Schliep, 2019), circlise (Gu et al., 2014), chorddiag (Flor, 2020), coin (Hothorn et al., 2008), hrbrthemes (Rudis, 2020), patchwork (Pedersen, 2020), phytools (Revell, 2012), picante (Kembel et al.,

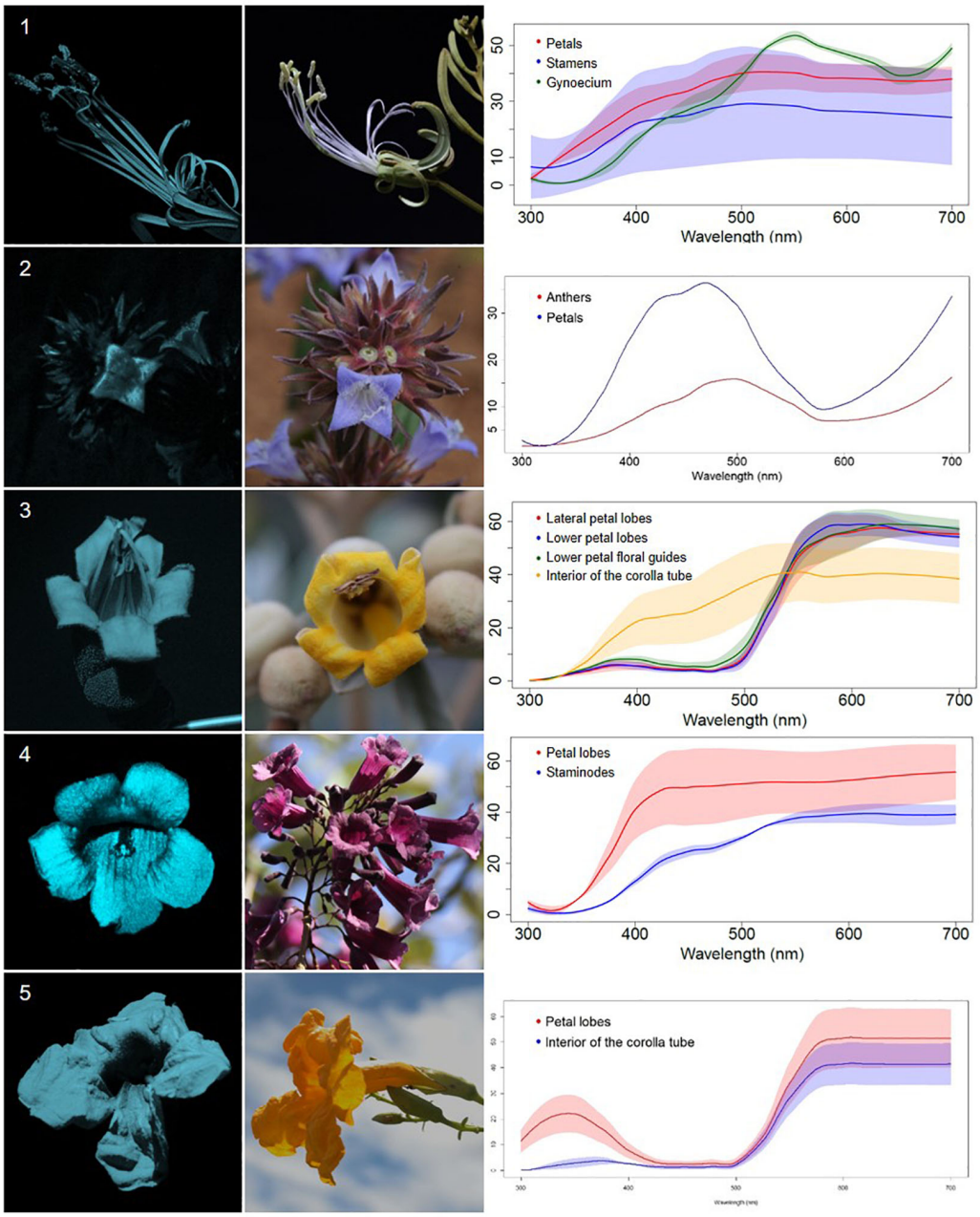


FIGURE 1 | UV-reflecting non-patterned flowers (R) from a Neotropical savanna community. Each row comprises information of one species. First column: UV-photography. Second column: flower as seen by the human eye (conventional photography). Third column: reflectance curves of different portions of the flowers. Row 1. *Bauhinia rufa* (Bong.) Steud. (Leguminosae). Row 2. *Spermacoce poaya* A.St.-Hil. (Rubiaceae). Row 3. *Zeyheria montana* Mart. (Bignoniaceae). Row 4. *Jacaranda caroba* (Vell.) DC. (Bignoniaceae). Row 5. *Adenocalymma peregrinum* (Miers) L.G.Lohmann (Bignoniaceae).

2010), rcompanion (Mangiafico, 2020), tidyverse (Wickham et al., 2019), viridis (Garnier, 2018).

RESULTS

We classified the 80 observed plant species, belonging to 68 genera, in 29 families, into five categories, based on floral UV features: (R) UV-reflecting; (A) UV-absorbing; (BE) bullseye; (CM) contrasting corolla markings oriented toward floral resources or reproductive structures (UV-reflecting markings in a flower with predominantly UV-absorbing corolla, and *vice versa*) and (CR) contrasting reproductive structures (UV-reflecting reproductive structures in a flower with UV-absorbing corolla, and *vice versa*) (**Figures 1–5**, **Supplementary Figure 1**, and **Supplementary Table 2**). It is noteworthy that 7.5% belonged

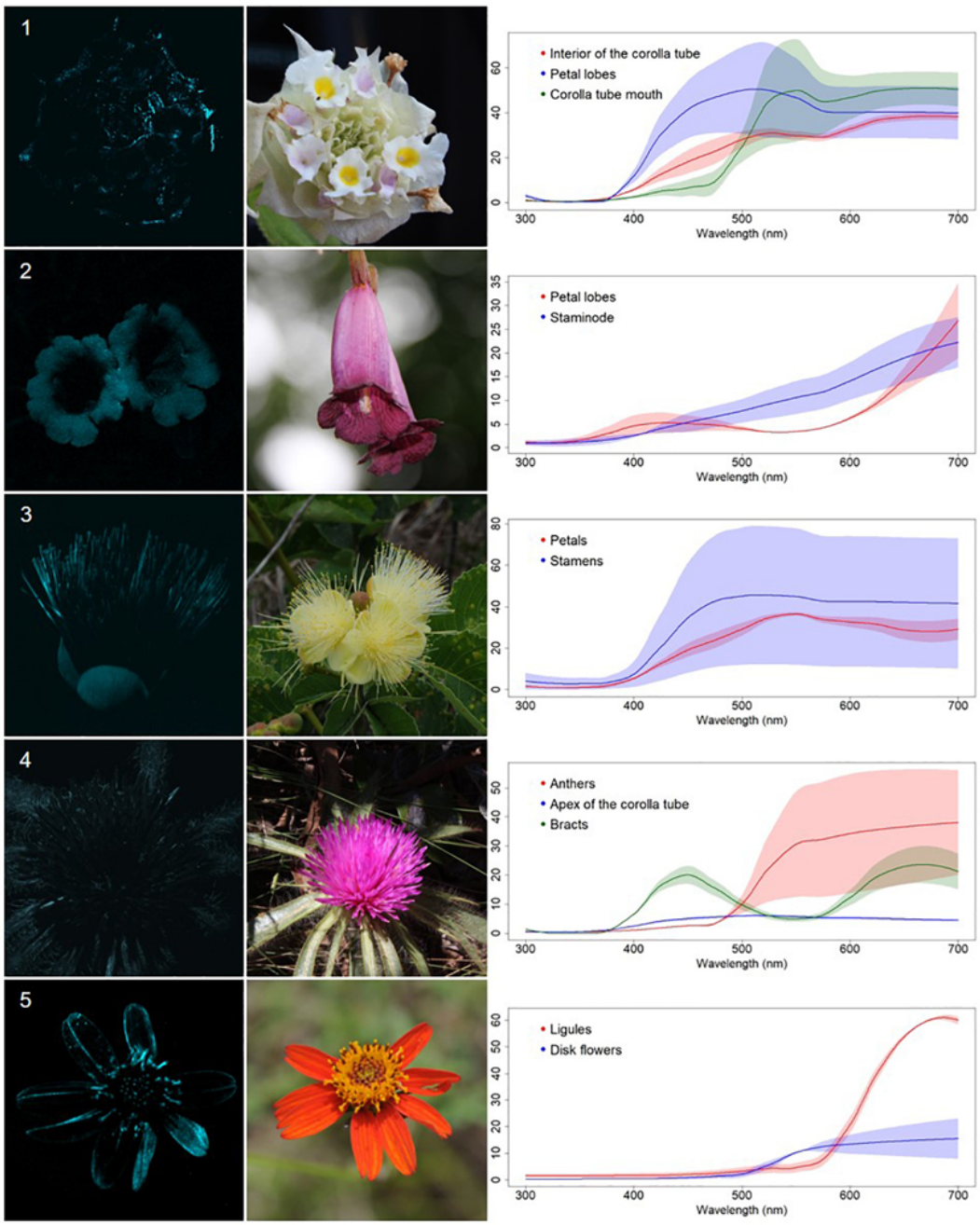


FIGURE 2 | UV-absorbing non-patterned flowers (A) from a Neotropical savanna community. Each row comprises information of one species. First column: UV-photography. Second column: flower as seen by the human eye (conventional photography). Third column: reflectance curves of different portions of the flowers. Row 1. *Lippia lupulina* Cham. (Verbenaceae). Row 2. *Jacaranda rufa* Silva Manso (Bignoniaceae). Row 3. *Caryocar brasiliense* A.St.-Hil. (Caryocaraceae). Row 4. *Gomphrena macrocephala* A.St.-Hil. (Amaranthaceae). Row 5. *Bidens gardnerii* Baker (Compositae).

to the (R) category and 58.75% to the (A) category, which indicates that 66.25% of species did not present any UV pattern but instead were homogeneously reflecting or absorbing UV (**Figure 6**). On the other hand, the remaining 33.75% of species presented UV floral patterns. We use the term UV-patterns here to refer to flowers that, differently from the two previous categories that were uniform in relation to UV properties, presented heterogeneous UV properties. These flowers showed a mixture of reflective and absorbing portions that creates various patterns within the attraction unit. These UV floral patterns could be considered as floral guides (*sensu* Dafni and Giurfa, 1999). From our sampled species, 8.75% presented (BE) pattern, 6.25% presented (CM) pattern and 18.75%, (CR) pattern (**Figure 6**).

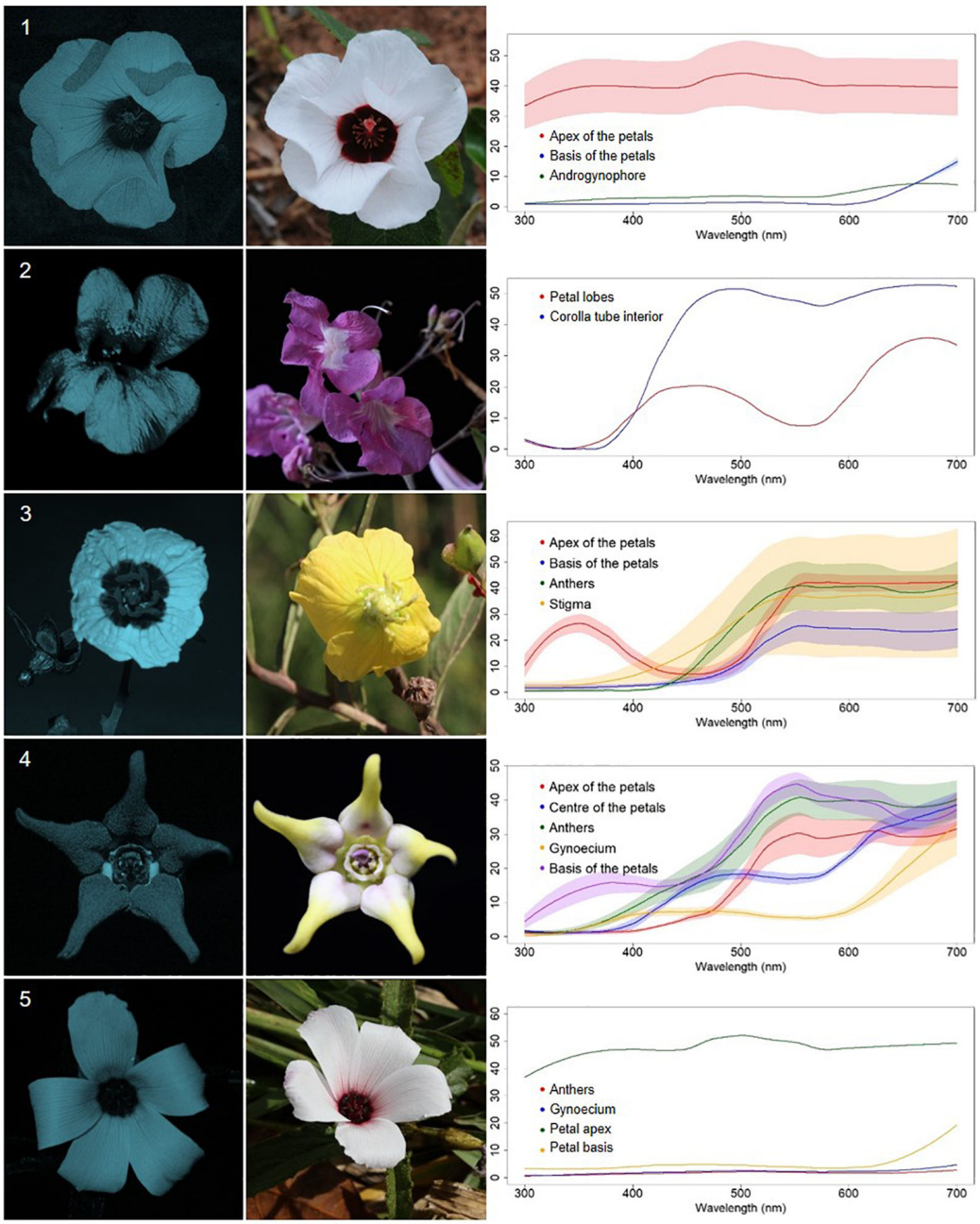


FIGURE 3 | Bullseye UV-patterned flowers (BE) from a Neotropical savanna community. Each row comprises information of one species. First column: UV-photography. Second column: flower as seen by the human eye (conventional photography). Third column: reflectance curves of different portions of the flowers. Row 1. *Peltaea polymorpha* (A. St.-Hil.) Krapov. and Cristóbal (Malvaceae). Row 2. *Cuspidaria* sp. (Bignoniaceae). Row 3. *Ludwigia nervosa* (Poir.) H.Hara (Onagraceae). Row 4. *Oxypetalum appendiculatum* Mart. (Apocynaceae). Row 5. *Piriqueta aurea* (Cambess.) Urb. (Turneraceae).

We observed a significant degree of phylogenetic signal between the floral UV features and plant species phylogeny ($\delta = 1.731154$; $p < 0.001$; **Figure 7**).

We observed that there was an association between the type of floral UV feature and pollination systems ($\chi^2 = 9.5294$; $p = 0.04915$; **Figure 8**). See **Supplementary Table 3** for percentages and detailed statistics. UV-reflecting flowers (R) were mainly associated with bee-pollination (66.6% of plant species), but also with hummingbird-, and bat-pollination. UV-absorbing flowers (A) were associated with almost all pollinator groups, except with fly-pollination and also presented high percentage of bee-pollinated species (40.4%). UV-absorbing flowers (A) were the only category associated with hawkmoth and butterfly-pollinated species (**Figure 4**). Regarding the plant species that

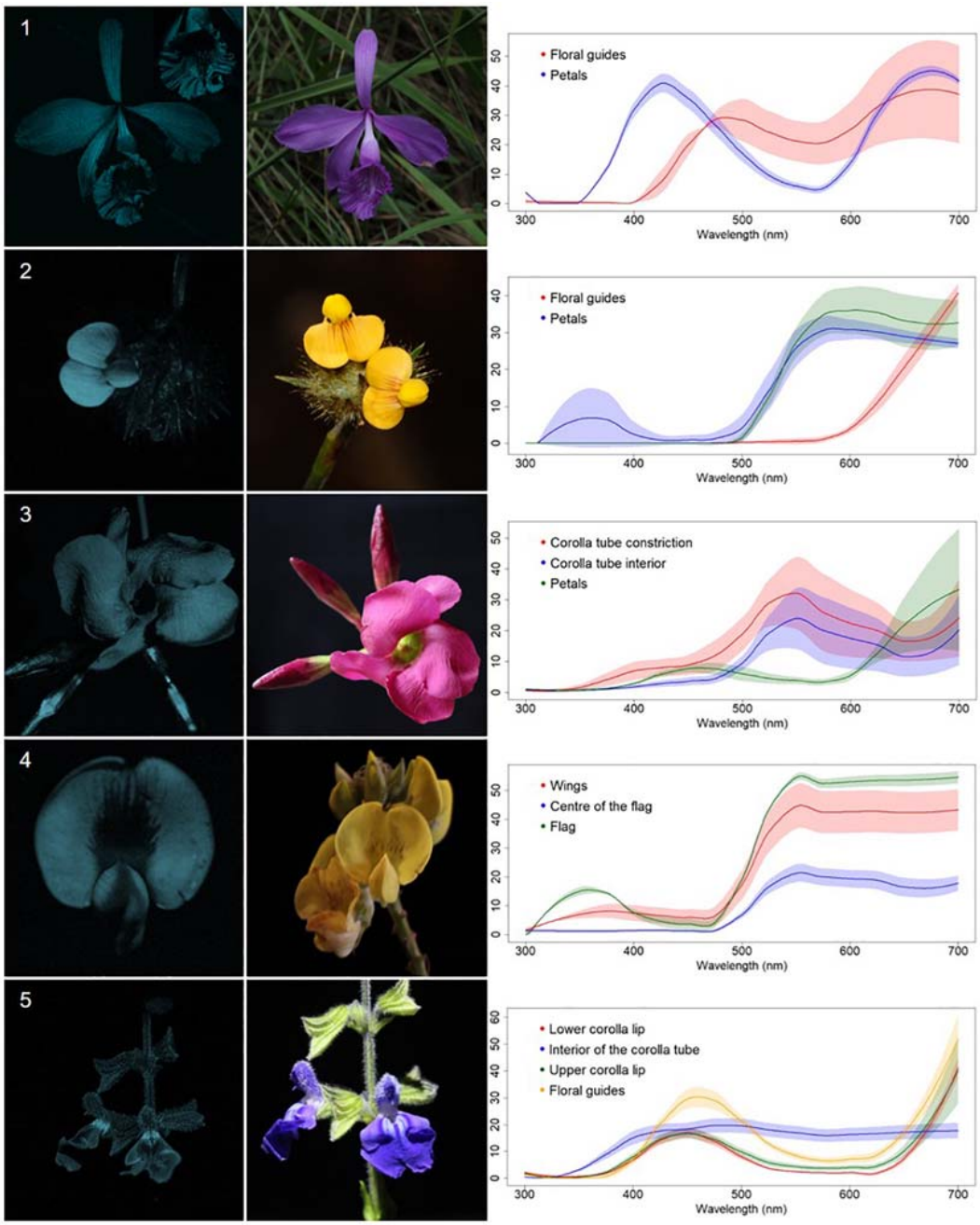


FIGURE 4 | Contrasting corolla markings oriented towards floral resources UV-patterned flowers (CM) from a Neotropical savanna community. Each row comprises information of one species. First column: UV-photography. Second column: flower as seen by the human eye (conventional photography). Third column: reflectance curves of different portions of the flowers. Row 1. *Epistephium sclerophyllum* Lindl. (Orchidaceae). Row 2. *Stylosanthes guianensis* (Aubl.) Sw. (Leguminosae). Row 3. *Temnadenia violacea* (Vell.) Miers (Apocynaceae). Row 4. *Crotalaria micans* Link (Leguminosae). Row 5. *Salvia minarum* Briq. (Lamiaceae).

presented floral UV-patterns, all three categories were mainly associated with bee-pollination. The species with a bullseye pattern (BE), besides being associated with bee-pollination (71.4%), were also associated with generalist pollination (28.6%) (**Figure 4**). It is noteworthy that flowers with contrasting markings in the corolla (CM) were exclusively associated with bee-pollination (**Figure 4**). Similarly, to the observed for (BE), (CR) species were associated primarily with bee-pollination (80.0%), but also with generalist pollination (6.6%) (**Figure 8**). (CR) was the only category that was associated with fly-pollination. See **Supplementary Tables 3**, **4** for percentages and detailed statistics.

We found an association between floral UV features and floral resources (χ^2 = 29.278; p < 0.0001; **Figure 9**).

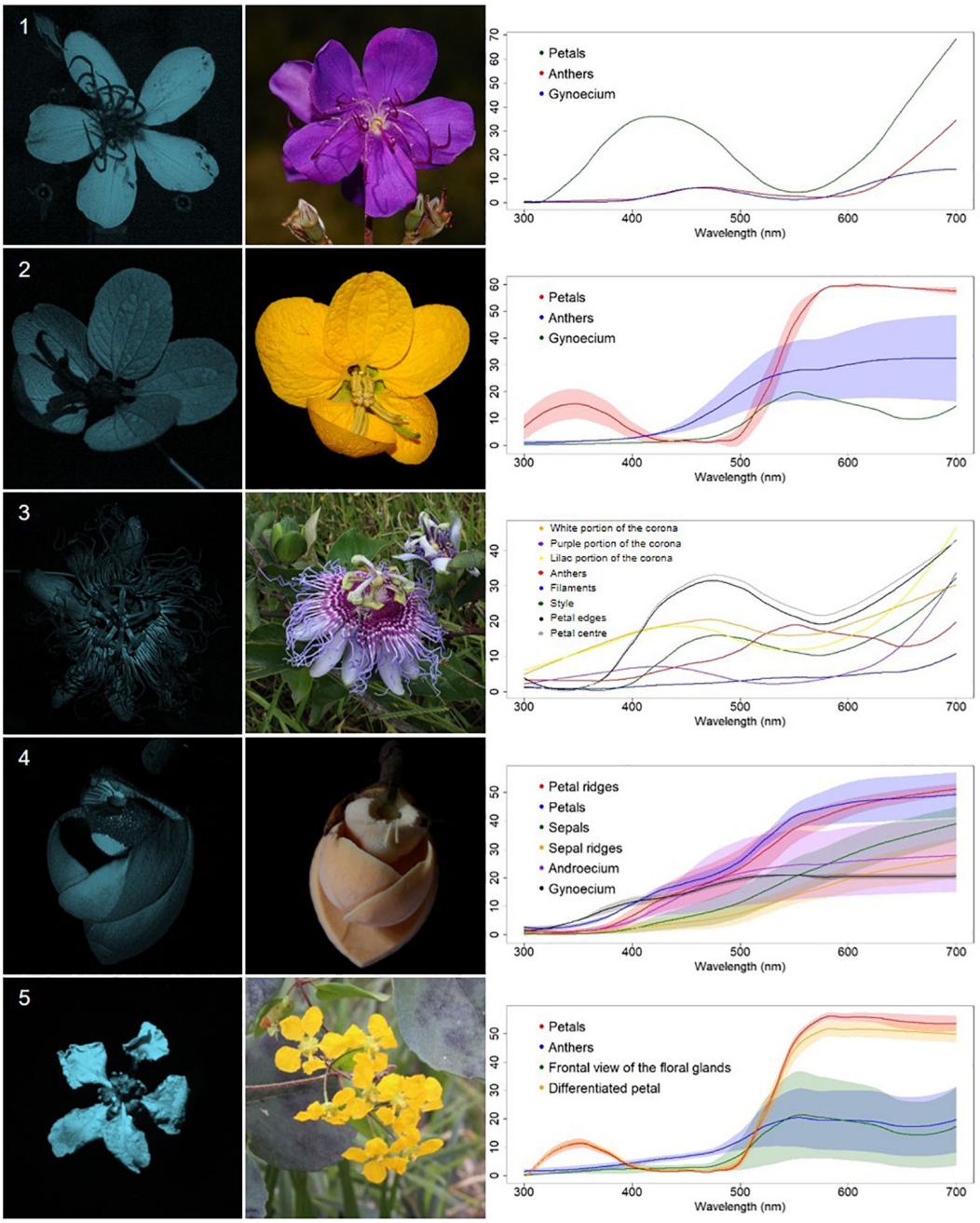


FIGURE 5 | Contrasting reproductive structures UV-patterned flowers (CR) from a Neotropical savanna community. Each row comprises information of one species. First column: UV-photography. Second column: flower as seen by the human eye (conventional photography). Third column: reflectance curves of different portions of the flowers. Row 1. *Pleroma stenocarpa* (DC.) Cogn. (Melastomataceae). Row 2. *Senna rugosa* (G.Don) H.S.Irwin and Barneby(Leguminosae). Row 3. *Passiflora cincinnata* Mast. (Passifloraceae). Row 4. *Annona coriacea* Mart. (Annonaceae). Row 5. *Janusia guaranitica* (A.St.-Hil.) A.Juss. (Malpighiaceae).

The most frequent floral resource observed was nectar, followed by pollen. (R), (A), (CM), and (BE) were similarly associated with high frequencies of plant species that have nectar as resource (from 70.0 to 100% of the plant species in each category). Indeed, (R) and (CM) had exclusively nectar as a resource. Species belonging to (BE) category also had pollen, and species from (A) category had also pollen and oil as resource (**Figure 9**). On the other hand, (CR) flowers differed from those categories and were associated primarily with pollen as resource (57.9%) and in a low frequency, with nectar (15.8%) (**Figure 9**). Additionally, (CR) species are the only ones to present tissue as a resource. See **Supplementary Tables 5**, **6** for percentages and detailed statistics.

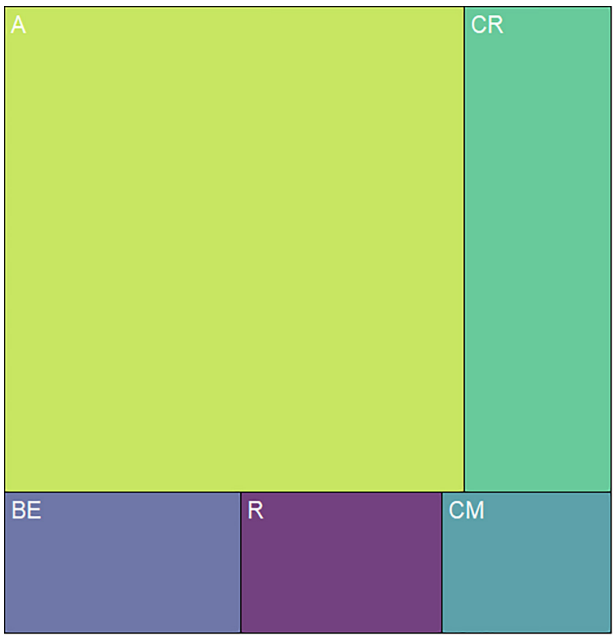


FIGURE 6 | Distribution of the 80 sampled species among the types of UV features. The relative area occupied by each quadrilateral polygon corresponds to the relative occurrence of each floral UV category. From the sampled species, 7.5% presented flowers that belonged to the (R) category and 58.7% to the (A) category, which indicates that 66.2% of species were non-UV-patterned. The remaining 33.8% of species presented UV floral patterns, being that 8.75% presented (BE) pattern, 6.25% presented (CM) pattern and 18.75%, (CR) pattern. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented towards floral resources; CR, contrasting reproductive structures.

We also observed that there was an association between type of floral UV feature and type of attraction unit (χ^2 = 12.33; p = 0.01506; **Figure 10**). There was an association between UV-absorbing species and the presence of inflorescences and intermediate attraction units. Additionally, (CR) and (CM) were only associated with flowers as attraction units. See **Supplementary Tables 7**, **8** for percentages and detailed statistics.

We found no association between floral UV features and floral non-UV colour features (χ^2 = 6.7744; p = 0.1483; **Figure 11**). See **Supplementary Table 9** for percentages and detailed statistics.

DISCUSSION

We observed that most of the Neotropical savanna plant species are UV-patternless, belonging to (R) and (A) categories. However, 33.3% of the species show a composition of UV- reflecting and UV-absorbing areas that create different UV-patterns. We also show that floral UV features are influenced by plant species relatedness. We found association between the floral UV features presented by plant species and their pollinators, floral resources and the attraction units. However, there was no association of floral UV features with the presence/absence of non-UV colour patterns.

Floral UV Features in Neotropical Savanna Plant Species

Besides being caused by floral nanoscale physical structures (Glover, 2014; Papiorek et al., 2014; Moyroud et al., 2017; van der Kooi et al., 2019), floral colour is a result of selective absorption or reflection of light caused by pigments (Kevan et al., 1996; van der Kooi et al., 2019). Thus, flowers can be homogeneously coloured or can present a combination of different colours, creating high contrast within the flower (Dafni and Kevan, 1996) and the enormous variety of patterns we observe in nature (Kevan et al., 1996; Schlangen et al., 2009). Despite the increase in contrasts created by colour patterns, our data shows a predominance of UV-patternless flowers, which means that almost 70% of species have pigments related to UV reflectance/absorbance uniformly distributed through the floral whorls (Glover, 2014).

On the other hand, 33.3% of the species presented UV-patterned flowers. We considered the three types of floral UV-patterns as "floral guides," following Dafni and Giurfa (1999) definition, without discriminating the resource involved. Bullseye patterned flowers (BE) usually present an absorbing centre surrounded by a reflecting portion, which is ascribed to a spatial separation of flavonoids that matches exactly the UV-pattern (Brehm and Krell, 1975). Interestingly, the reproductive structures of all types of UV-patterned flowers are mostly located in UV-absorbing parts of the flowers. The species presenting contrasting marking pattern (CM) show both UV-absorbing or UV-reflecting markings converging at the region where the resource and reproductive structures are located. Also, most species showing contrasting reproductive structures (CR) have UV-absorbing androecium and gynoecium. In fact, UV-absorbing pigments, such as flavonoids, protect plants against UV-B radiation (Lee and Gould, 2002; Agati and Tattini, 2010; Landi et al., 2015). Anthers walls of some species may filter up to 98% of the UV-B radiation that reaches the pollen grains (Flint and Caldwell, 1983). So, UV-absorbing reproductive structures have been associated with pollen protection (Lunau, 2000 and references therein), since the high incidence of these wavelengths can decrease pollen production and viability (Demchik and Day, 1996; but see Peach et al., 2020). In general, flowers from savanna, a tropical open vegetation, are exposed to a high sunlight incidence (Pfadenhauer and Klötzli, 2020). So, UV-absorbing pigments in reproductive structures may represent an important trait for pollen protection, especially in the context of ozone layer degradation and UV incidence increase (Koski et al., 2020). In addition, the uniformly UV-absorbing corolla of the (A) category may provide extra protection, reducing the reflection of UV-light from petals to the anthers (Koski et al., 2020) and sheltering anthers since the bud stage (Flint and Caldwell, 1983).

Bees tend to make the first physical contact with a flower by means of an antennal reaction at an area of the flower displaying high chromatic contrast and high colour purity (Lunau et al., 1996; Rohde et al., 2013). Therefore, besides gamete protection, the UV-absorbing centre creates a centripetal



FIGURE 7 | Phylogenetic tree representing 80 Neotropical savanna plant species based on the megaphylogeny by Qian and Jin (2016), including the floral UV features of each plant species. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented toward floral resources; CR, contrasting reproductive structures. The types of floral UV features are represented by symbols in which blue represents UV-reflection and black represents UV-absorption. *Pleroma stenocarpa* and *Betencoutia scarlatina* are represented in the figure by their synonyms *Tibouchina stenocapa* and *Camptosema scarlatinum*, respectively, because the updated nomenclature was not compatible with the species name in the megaphylogeny.

increased gradient of chromatic contrast and colour purity, triggering bees' preferences for colours of high spectral purity (Lunau, 1991a,b; Dafni and Giurfa, 1999). In addition to a marked contrast within the flower, floral UV-patterns may increase the contrast between the flower and the leaf-background, which usually absorbs ultraviolet light (Kevan and Backhaus, 1998; Camargo et al., 2014; van der Kooi et al., 2019).

Floral UV Features' Phylogenetic Signal

Flowers of closely related species from the Neotropical savanna presented similar floral UV features. Other studies also found phylogenetic restrictions related to UV-patterns (Koski and Ashman, 2016; Koski, 2020). In general, flower main colour is considered a labile floral trait, presenting weak or even no phylogenetic signal (McEwen and Vamosi, 2010; Shrestha et al., 2014; Muchhala et al., 2014; Bergamo et al., 2018). However, we must consider that flower colour patterns may be associated with the general flower structure (E-Vojtkó et al., 2020), such as resource location, reproductive structures position and exposure, which are probably more phylogenetically conservative traits. Indeed, we confirmed that floral UV features, although being an important signal for pollinators (Daumer, 1958; Koski and Ashman, 2014; Papiorek et al., 2016), present phylogenetic signal, which means that the phylogenetic relatedness of the plant species can help predict their UV-features.

Floral UV Features and Plant-Pollinator Interaction

Evidence of floral colour and colour pattern importance for plant-pollinator interaction has been accumulated since the systematic observations by Sprengel (1793). In fact, in the present study, we

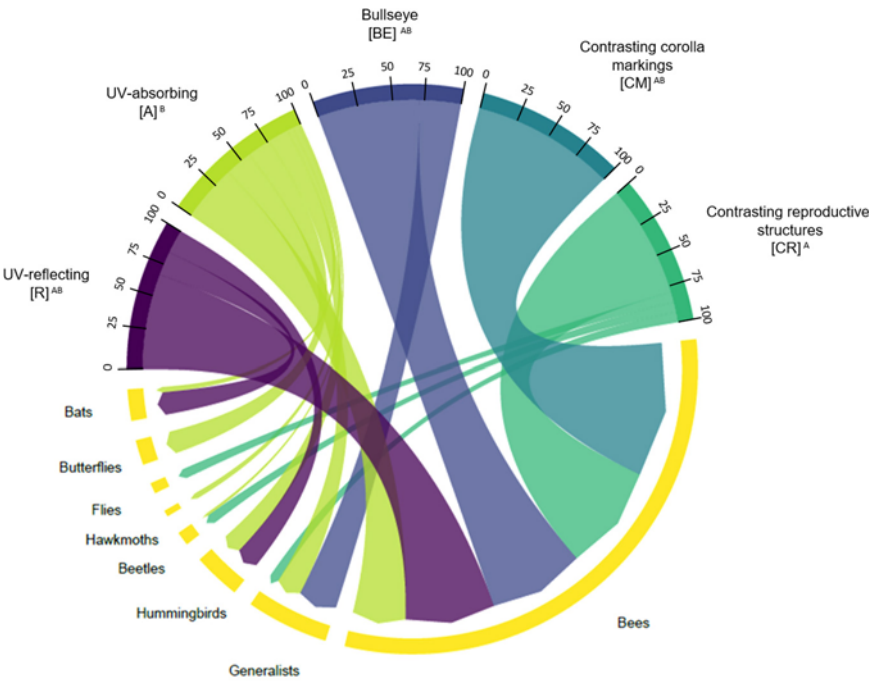


FIGURE 8 | Chord diagram showing the relationship between the types of floral UV features and pollination systems of 80 plant species. The chords are unidirectional. Chord thickness corresponds to the percentage of species with each floral UV feature that is related to a given pollination system. Similar letters indicate that the UV categories were related to similar proportions of each pollination system (χ^2 = 9.5294; p = 0.04915). See **Supplementary Tables 3**, **4** for percentages and detailed statistics. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented towards floral resources; CR, contrasting reproductive structures.

observed a clear association between the type of floral UV feature and pollinator groups. This result could indicate that pollinators present preferences for specific types of floral UV features, which could lead to pollinators acting as selection agents upon this floral trait. Understanding the real role of pollinators in the angiosperm diversification process is a great challenge (van der Niet and Johnson, 2012). However, it is important to consider that the correlations of floral patterns with other floral traits, and their ecological role may also influence their phylogenetic distribution (Koski, 2020).

Here we show that both uniform floral UV categories, i.e., the UV-patternless flowers, are associated with an ample variety of pollination systems, being (A) associated with all pollinator groups, except flies. We must highlight that these flowers may present colour patterns related to human-visible colours, which are also important signals to different pollinators, such as bees, butterflies, and flies (Lunau et al., 1996; Kandori and Ohsaki, 1998; Heuschen et al., 2005; Hansen et al., 2012). Even though there are some simple connections between UV features and colours that are visible to humans, such as the fact that some UV-absorbing flavonoids also contribute to the yellow colour of Asteraceae and Leguminosae flowers, being called "yellow flavonols" (Harborne, 1967). However, carotenoids are also responsible for the flower's yellow colour but are UV-reflecting (Kevan et al., 1996 and references therein; Schlangen et al., 2009). Therefore, making direct relations between human visible colours and UV-features is not as straightforward as it could seem, because the same colour in human vision can present different UV properties. Chittka et al. (1994), using a large sample size, showed that most flowers (∼74%) were UV-absorbing and the minority (∼26%) were UV-reflecting, regardless of what colour they displayed in the human-visible spectra. In the present study, we found a similar predominance of UV-absorbing flowers. Approximately 90% of the non-patterned flowers are UV-absorbing, while the remaining 10% are UV-reflective.

Even though flower's colour contrast against the background is crucial for pollinators to locate a flower from a distance, floral colour patterns (floral guides), such as the ones presented by the (BE), (CM), and (CR) patterns, can act at short-distance pollinator orientation cues, mainly for insects (Manning, 1956; Lunau et al., 1996; Rohde et al., 2013). These patterns' importance is ascribed to the fact that pollinators use them to discriminate among flowers from different species and to learn to obtain resources in a more accurate manner, which may represent less energy and time spent by pollinators (Kevan et al., 1996; Leonard and Papaj, 2011; Glover, 2014; de Jager et al., 2017), and higher plant fitness (Hansen et al., 2012). It is remarkable that in our study, these floral UV-patterns, (BE), (CM), and (CR) were associated almost exclusively with bee- and generalist-pollination, which for most plant species includes bees as pollinators (see Gottsberger and Silberbauer-Gottsberger, 2006).

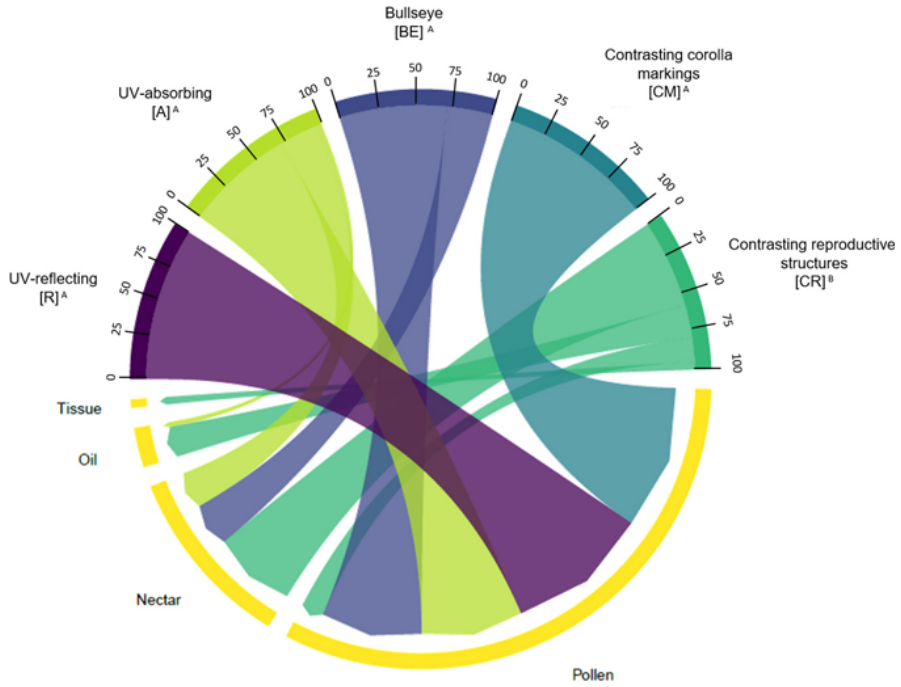


FIGURE 9 | Chord diagram showing the relationship between the types of floral UV features and the type of floral resource of 80 plant species. The chords are unidirectional. Chord thickness corresponds to the percentage of species with each UV feature that is related to a given floral resource type. Similar letters indicate that the UV categories were related to similar proportions of each floral resource type ($\chi^2 = 29.278$; $p = 6.862 \times 10^{-6}$). See **Supplementary Tables 5**, **6** for percentages and detailed statistics. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented towards floral resources; CR, contrasting reproductive structures.

The external reflexive portion of the bullseye inflorescences may be used as a landing platform by pollinators, in contrast, the UV-absorbing centre of the inflorescence acts as a floral guide (Daumer, 1956, 1958). Such floral guide is perceived by bees, which show probing responses at the boundary between UV-reflecting and UV-absorbing floral regions (Daumer, 1958; Burkhardt et al., 1967; Papiorek et al., 2016). In fact, studies show that UV-patterned flowers may be more conspicuous to insects than non-patterned (Koski and Ashman, 2014; Papiorek et al., 2016) and that the presence of floral patterns may increase bees' floral constancy (Scora, 1964). Bees can also learn which floral pattern is more profitable and change visiting behaviour (Xu and Plowright, 2017). So, it has been shown in several ways that floral UV-patterns can also influence pollinator choice and behaviour (Peterson et al., 2015; Brock et al., 2016; Papiorek et al., 2016; Koski, 2020), like colour patterns in human-visible wavelengths.

All the hummingbird-pollinated species in our study were UV-patternless, being most of them UV-absorbing. Our results are consistent with other studies that show that patterned flowers are less common in hummingbird-pollinated species than in bee-pollinated ones (Proctor and Yeo, 1973; Faegri and Van der Pijl, 1979; Camargo et al., 2019). Additionally, the absence of floral guides, coupled with UV-absorbing red flowers is seen as achromatic by bees, which creates a private niche for hummingbirds to explore these flowers without competing with bees for floral resources (Lunau et al., 2011; Papiorek et al., 2013; Camargo et al., 2019). This can be observed in 87.5% of the hummingbird-pollinated species in our study. The only species UV-reflecting (R) do not present any colour trait specifically related to hummingbird-pollination.

It is noteworthy that most nocturnal insect-pollinated species in our study are UV-absorbing (A). These species were pollinated by hawkmoths and beetles, which present colour vision even in moonless nights (Johnsen et al., 2006; Goyret et al., 2008; Théry et al., 2008) and eyes with high light sensitivity but low spatial resolution (Hempel de Ibarra et al., 2015). They also show slow image processing, which can affect insect behaviour, especially regarding flight speed and trajectory (Sponberg et al., 2015). In this context, one could expect that the presence of floral colour patterns, regardless of the wavelength, would be hard to distinguish by nocturnal insect pollinators. However, there is evidence that hawkmoths can perceive UV bullseye patterns in dim light (Hirota et al., 2019). Bats use different sensory cues for perceiving the surrounding environment and may constantly integrate information obtained through echolocation and vision (Boonman et al., 2013). They have S opsin genes that are sensitive to UV light, enabling them to use this colour especially at dawn and at dusk, when UV light is relatively more abundant (Wang et al., 2004). There is growing evidence of the general UV-sensitivity in bats and Gorresen et al. (2015) bring solid corroboration of widespread dim-light UV vision in bats. So, the corolla and androecium UV-reflecting (R) in *Bauhinia rufa*, a bat-pollinated species, may represent important cues for flower location by this pollinator group.

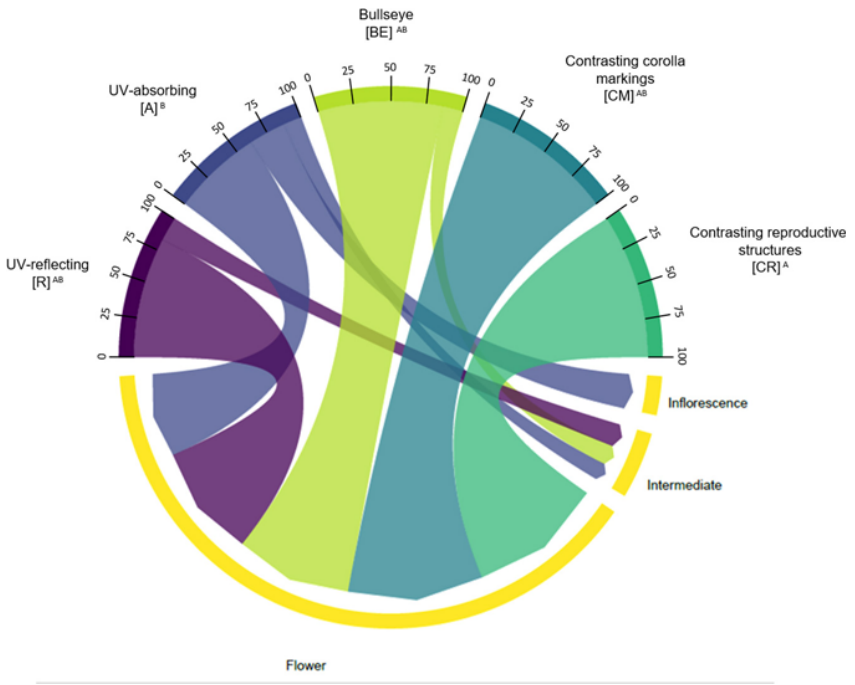


FIGURE 10 | Chord diagram showing the relationship between the types of floral UV features and the type of attraction unit of 80 plant species. The chords are unidirectional. Chord thickness corresponds to the percentage of species with each UV feature that is related to a given attraction unit. Flowers within each UV category presented different percentages of species with each attraction unit type ($\chi^2 = 12.33$; $p = 0.01506$). See **Supplementary Tables 7**, **8** for percentages. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented towards floral resources; CR, contrasting reproductive structures.

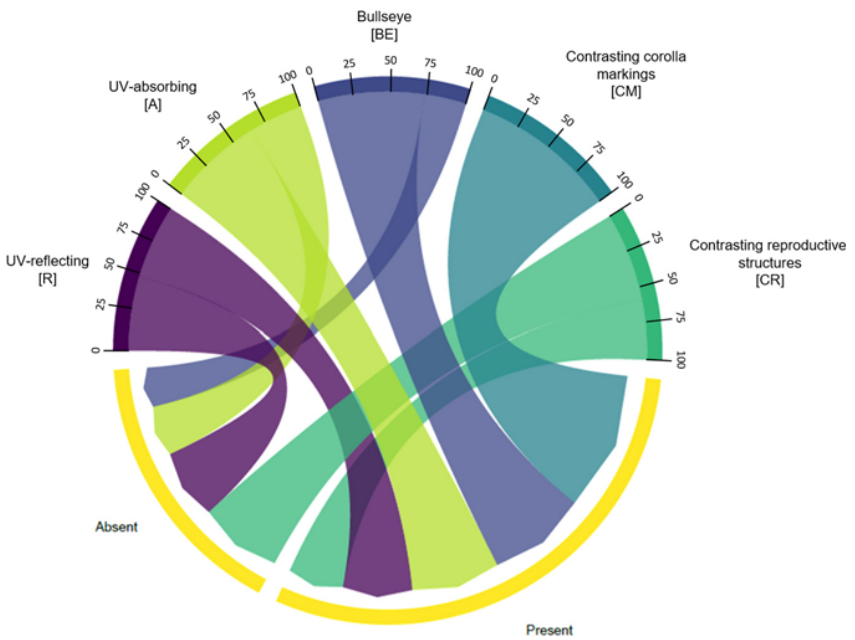


FIGURE 11 | Chord diagram showing the relationship between the types of floral UV features and the presence/absence of non-UV colour patterns of 80 plant species. The chords are unidirectional. Chord thickness corresponds to the percentage of species with UV features that is related to a presence/absence of non-UV colour pattern. Flowers within each UV category presented similar percentages of species with presence/absence of non-UV colour patterns ($\chi^2 = 6.7744$; $p = 0.1483$). See **Supplementary Table 9** for percentages. R, non-patterned UV-reflecting; A, non-patterned UV-absorbing; BE, bullseye; CM, contrasting corolla markings oriented towards floral resources; CR, contrasting reproductive structures.

The sampled butterfly-pollinated species in our study were non-patterned, UV-absorbing (A) and most of them presented patterns in the human-visible wavelengths. Even though butterflies are mainly associated with patterned flowers (Proctor and Yeo, 1973; Kandori and Ohsaki, 1998), they can perceive flowers in nature regardless of the presence of UV patterns or uniform UV reflectance. Especially because butterflies present a high capability to discriminate colours from UV to deep red (Stavenga and Arikawa, 2006). On the other hand, even with an unclear colour preference, flies are usually related to yellowish flowers (Lunau, 2014), such as the (EC) fly-pollinated species registered in our study. The absence of UV reflectance does not discourage fly-pollination of the tropical *Hypoxis camerooniana* (Klomberg et al., 2019) but the presence of UV pattern favoured fly visitation of the temperate *Argentina anserina* flowers. Flies are sensitive to UV (Chittka and Briscoe, 2001; Cronin et al., 2014; Lunau, 2014) and maybe tuned to yellow UV-patterned flowers (An et al., 2018), similar to *Cissus erosa*, the fly-pollinated species sampled here.

In the present study, we verified that there is an association between the type of floral UV feature and floral resources. Both UV-patternless categories, (R) and (A), and two patterned categories, (BE) and (CM), are mostly associated with nectar. Nectar is the most common trophic resource exploited by virtually all pollinator groups (Nepi, 2017; Nepi et al., 2018). The separation observed here shows some connections among floral UV features, the type of floral resource and the pollinator group that exploit it, since butterflies, hawkmoths, bats, and hummingbirds are associated with non-patterned flowers, (R) and (A), which, in turn, present mainly nectar as a resource. (BE) and (CM) are mainly associated with bees and other insects that also search for nectar. The (CM) category presented a mixture of species, with some reflecting and others absorbing UV at the lines, showing UV-absorbing lines that can act as an honest signal (Pélabon et al., 2012; Lunau et al., 2020). (CR) species differ from all the other categories, by being mainly associated with pollen as floral resource, which is exploited by social and solitary bees for adult and larval provisions (Cane and Tepedino, 2017). Additionally, this floral UV category could be related with resource signalling, since yellow and UV-absorbing pollen and anthers (or mimics) trigger behavioural responses in bees and flies (Lunau et al., 2017 and references therein). Interestingly, oil-collecting bees are sensitive to floral visual changes (Ferreira and Torezan-Silingardi, 2013; Melo et al., 2018) and half of the Malpighiaceae species (mainly having oil as resource) show UV-absorbing reproductive structures (CR), a pattern usually associated with pollen-flowers (Lunau et al., 2017).

Individual flowers can present all the types of UV-features considered here, but inflorescences were always UV-absorbing. To our knowledge, this is the first study that analyses the relationship between colour patterns and the unit of attraction and further studies are necessary considering these arrangements in experiments testing for pollinators' perception. Although we found no association between the presence/absence of non-UV colour patterns and UV features, most of the inflorescences that were UV-absorbing showed colour patterns in other wavebands. These patterns could be created within a flower or among flowers within inflorescences, like observed here for many Verbenaceae species.

In general, it has been shown that conspicuous flowers can receive more pollinator visits (Chittka et al., 2001), which might represent selection pressures toward more contrasting and distinguished visual features. Indeed, floral UV-patterns may favour bee attraction (Rae and Vamosi, 2013; Klomberg et al., 2019) and may increase the probability of the pollinator reaching floral resources, reducing handling time (Dinkel and Lunau, 2001; Leonard and Papaj, 2011). In spite of the predominance of UV-patternless flowers in butterfly-, hummingbird-, beetle-, bat-, and hawkmoth-pollinated species, we cannot disregard the importance of UV patterns for these pollinators until there are experimental evidences. Additionally, we confirmed that floral UV features, although being reportedly important signal for pollinators, are phylogenetically constrained. Therefore, the distribution of floral UV-features in nature cannot be ascribed to a single ecological or evolutionary factor. Thus, we still need to study wider assemblages of plant species, with different pollination systems to test some evolutionary hypotheses and to gain a broader knowledge of the selective pressures operating on UV features. In this context, adding data from a globally widespread biome, such as savanna, can bring a deeper understanding of ecological and evolutionary processes involved in floral UV feature diversification.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

EG and PT conceptualised and designed the study. PT organised the database and performed the statistical analysis. EG, PT, and MC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.618028/full#supplementary-material

REFERENCES

Abrahamson, W. G., and McCrea, K. D. (1977). Ultraviolet light reflection and absorption patterns in populations of Rudbeckia (Compositae). *Rhodora* 79, 269–277.

Agati, G., and Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection. *New Phytol.* 186, 786–793. doi: 10.1111/j.1469-8137.2010.03269.x

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly *Eristalis tenax* prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 221(Pt 22):jeb184788. doi: 10.1242/jeb.184788

Bergamo, P. J., Telles, F. J., Arnold, S. E., and de Brito, V. L. G. (2018). Flower colour within communities shifts from overdispersed to clustered along an alpine altitudinal gradient. *Oecologia* 188, 223–235. doi: 10.1007/s00442-018-4204-5

Boonman, A., Bar-On, Y., and Yovel, Y. (2013). It's not black or white—on the range of vision and echolocation in echolocating bats. *Front. Physiol.* 4:248. doi: 10.3389/fphys.2013.00248

Borges, R., Machado, J. P., Gomes, C., Rocha, A. P., and Antunes, A. (2019). Measuring phylogenetic signal between categorical traits and phylogenies. *Bioinformatics.* 35, 1862–1869. doi: 10.1093/bioinformatics/bty800

Bradshaw, H. D., and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426, 176–178. doi: 10.1038/nature02106

Brehm, B. G., and Krell, D. (1975). Flavonoid localization in epidermal papillae of flower petals: a specialized adaptation for ultraviolet absorption. *Science* 190, 1221–1223. doi: 10.1126/science.190.4220.1221

Brock, M. T., Lucas, L. K., Anderson, N. A., Rubin, M. J., Cody Markelz, R. J., Covington, M. F., et al. (2016). Genetic architecture, biochemical underpinnings and ecological impact of floral UV patterning. *Mol. Ecol.* 25, 1122–1140. doi: 10.1111/mec.13542

Bukovac, Z., Dorin, A., Finke, V., Shrestha, M., Garcia, J., Avargues-Weber, A., et al. (2017). Assessing the ecological significance of bee visual detection and colour discrimination on the evolution of flower colours. *Evol. Ecol.* 31, 153–172. doi: 10.1007/s10682-016-9843-6

Burdon, R. C., Raguso, R. A., Gegear, R. J., Pierce, E. C., Kessler, A., and Parachnowitsch, A. L. (2020). Scented nectar and the challenge of measuring honest signals in pollination. *J. Ecol.* 108, 2132–2144. doi: 10.1111/1365-2745.13432

Burkhardt, D., Schleidt, W., and Altner, H. (1967). *Signals in the Animal World*. Crows Nest, NSW: George Allen & Unwin.

Camargo, M. G. G., Cazetta, E., Morellato, L. P. C., and Schaefer, H. M. (2014). Characterizing background heterogeneity in visual communication. *Basic Appl. Ecol.* 15, 326–335. doi: 10.1016/j.baae.2014.06.002

Camargo, M. G. G., Lunau, K., Batalha, M. A., Brings, S., de Brito, V. L. G., and Morellato, L. P. C. (2019). How flower colour signals allure bees and hummingbirds: a community-level test of the bee avoidance hypothesis. *New Phytol.* 222, 1112–1122. doi: 10.1111/nph.15594

Cane, J. H., and Tepedino, V. J. (2017). Gauging the effect of honey bee pollen collection on native bee communities. *Conserv. Lett.* 10, 205–210. doi: 10.1111/conl.12263

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543.

Chittka, L., Spaethe, J., Schmidt, A., and Hickelsberger, A. (2001). "Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision," in *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*, eds L. Chittka and J. D. Thomson (Cambridge: Cambridge University Press), 106–126. doi: 10.1017/cbo9780511542268.007

Chittka, L., and Briscoe, A. (2001). "Why sensory ecology needs to become more evolutionary - insect color vision as a case in point," in *Ecology of Sensing*, eds F. G. Barth and A. Schmid (Heidelberg: Springer), 19–37. doi: 10.1007/978-3-662-22644-5_2

Chittka, L., and Thomson, J. D. (2001). *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*. Cambridge: Cambridge University Press.

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Cronin, T. W., Johnsen, S., Marshall, N. J., and Warrant, E. J. (2014). *Visual Ecology*. Princeton: Princeton University Press.

Dafni, A., and Giurfa, M. (1999). "The functional ecology of floral guides in relation to insects behaviour and vision," in *Evolutionary Theory and Processes: Modern Perspectives*, ed. L. Blaustein (Dordrecht: Springer), 363–383. doi: 10.1007/978-94-011-4830-6_23

Dafni, A., and Kevan, P. G. (1996). Floral symmetry and nectar guides: ontogenetic constraints from floral development, colour pattern rules and functional significance. *Bot. J. Linn. Soc.* 120, 371–377. doi: 10.1111/j.1095-8339.1996.tb00487.x

Daumer, K. (1956). Reizmetrische untersuchung des Farbensehens der Bienen. *Z. Vergl. Physiol.* 38, 413–478.

Daumer, K. (1958). Blumenfarben, wie sie die Bienen sehen. *Z. Vergl. Physiol.* 41, 49–110.

de Jager, M. L., Willis-Jones, E., Critchley, S., and Glover, B. J. (2017). The impact of floral spot and ring markings on pollinator foraging dynamics. *Evol. Ecol.* 31, 193–204. doi: 10.1007/s10682-016-9852-5

Demchik, S. M., and Day, T. A. (1996). Effect of enhanced UV-B radiation on pollen quantity, quality, and seed yield in Brassica rapa (Brassicaceae). *Am. J. Bot.* 83, 573–579. doi: 10.2307/2445915

Dinkel, T., and Lunau, K. (2001). How drone flies (*Eristalis tenax* L., *Syrphidae, Diptera*) use floral guides to locate food sources. *J. Insect Physiol.* 47, 1111–1118. doi: 10.1016/s0022-1910(01)00080-4

E-Vojtkó, A., de Bello, F., Durka, W., Kühn, I., and Götzenberger, L. (2020). The neglected importance of floral traits in trait-based plant community assembly. *J. Veg. Sci.* 31, 529–539. doi: 10.1111/jvs.12877

Faegri, K., and Van der Pijl, L. (1979). *The Principles of Pollination Ecology*, 3rd Edn. Oxford: Pergamom Press.

Ferreira, C. A., and Torezan-Silingardi, H. M. (2013). Implications of the floral herbivory on Malpighiacea plant fitness: visual aspect of the flower affects the attractiveness to pollinators. *Sociobiology* 60, 323–328.

Flint, S. D., and Caldwell, M. M. (1983). Influence of floral optical properties on the ultraviolet radiation environment of pollen. *Am. J. Bot.* 70, 1416–1419. doi: 10.1002/j.1537-2197.1983.tb07931.x

Flor, M. (2020). *chorddiag: Interactive Chord Diagrams. R package version 0.1.2*. Available online at: http://github.com/mattflor/chorddiag/ (accessed March 25, 2020)

Garnier, S. (2018). *viridis: Default Color Maps from 'matplotlib'. R package version 0.5.1*. Available online at: https://CRAN.R-project.org/package=viridis (accessed March 25, 2020).

Glover, B. (2014). *Understanding Flowers and Flowering. Second Edition*. Oxford: Oxford University Press.

Goldsmith, T. H., and Goldsmith, K. M. (1979). Discrimination of colors by the black-chinned hummingbird, *Archilochus alexandri*. *J. Comp. Physiol.* 130, 209–220. doi: 10.1007/bf00614607

Goodale, E., Kim, E., Nabors, A., Henrichon, S., and Nieh, J. C. (2014). The innate responses of bumble bees to flower patterns: separating the nectar guide from the nectary changes bee movements and search time. *Naturwissenschaften* 101, 523–526. doi: 10.1007/s00114-014-1188-9

Gorresen, P. M., Cryan, P. M., Dalton, D. C., Wolf, S., and Bonaccorso, F. J. (2015). Ultraviolet vision may be widespread in bats. *Acta Chiropterol.* 17, 193–198. doi: 10.3161/15081109acc2015.17.1.017

Gottsberger, G., and Silberbauer-Gottsberger, I. (2006). *Life in the Cerrado: Pollination and Seed Dispersal*, Vol. 2. Ulm: Reta Verlag.

Goyret, J., Pfaff, M., Raguso, R. A., and Kelber, A. (2008). Why do Manduca sexta feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57, 761–780. doi: 10.1146/annurev.arplant.57.032905.105248

Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). *circlize* implements and enhances circular visualization in R. *Bioinformatics.* 30, 2811–2812. doi: 10.1093/bioinformatics/btu393

Hansen, D. M., Van der Niet, T., and Johnson, S. D. (2012). Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proc. R. Soc. B Biol. Sci.* 279, 634–639. doi: 10.1098/rspb.2011.1349

Harborne, J. B. (1967). Comparative biochemistry of the flavonoids-IV.: correlations between chemistry, pollen morphology and systematics in the family Plumbaginaceae. *Phytochemistry* 6, 1415–1428. doi: 10.1016/s0031-9422(00)82884-8

Harborne, J. B. (1981). Two gossypetin methyl ethers as ultraviolet patterning guides in the flowers of *Coronilla valentina*. *Phytochemistry* 20, 1117–1119. doi: 10.1016/0031-9422(81)83038-5

Hempel de Ibarra, N., Langridge, K. V., and Vorobyev, M. (2015). More than colour attraction: behavioural functions of flower patterns. *Curr. Opin. Insect Sci.* 12, 64–70. doi: 10.1016/j.cois.2015.09.005

Herrera, G., Zagal, J. C., Diaz, M., Fernández, M. J., Vielma, A., Cure, M., et al. (2008). Spectral sensitivities of photoreceptors and their role in colour discrimination in the green-backed firecrown hummingbird (*Sephanoides sephaniodes*). *J. Comp. Physiol. A* 194:785. doi: 10.1007/s00359-008-0349-8

Heuschen, B., Gumbert, A., and Lunau, K. (2005). A generalised mimicry system involving angiosperm flower colour, pollen and bumblebees' innate colour preferences. *Plant Syst. Evol.* 252, 121–137. doi: 10.1007/s00606-004-0249-5

Hill, P. S., Wells, P. H., and Wells, H. (1997). Spontaneous flower constancy and learning in honey bees as a function of colour. *Anim. Behav.* 54, 615–627. doi: 10.1006/anbe.1996.0467

Hirota, S. K., Miki, N., Yasumoto, A. A., and Yahara, T. (2019). UV bullseye contrast of *Hemerocallis* flowers attracts hawkmoths but not swallowtail butterflies. *Ecol. Evol.* 9, 52–64. doi: 10.1002/ece3.4604

Horth, L., Campbell, L., and Bray, R. (2014). Wild bees preferentially visit *Rudbeckia* flower heads with exaggerated ultraviolet absorbing floral guides. *Biol. Open* 3, 221–230. doi: 10.1242/bio.20146445

Hothorn, T., Hornik, K., van de Wiel, M. A., and Zeileis, A. (2008). Implementing a class of permutation tests: the coin package. *J. Stat. Softw.* 28, 1–23. doi: 10.18637/jss.v028.i08

Jogesh, T., Overson, R. P., Raguso, R. A., and Skogen, K. A. (2017). *Herbivory* as an important selective force in the evolution of floral traits and pollinator shifts. *AoB Plants* 9:lw088.

Johnsen, S., Kelber, A., Warrant, E., Sweeney, A. M., Widder, E. A., Lee, R. L., et al. (2006). Crepuscular and nocturnal illumination and its effects on color perception by the nocturnal hawkmoth *Deilephila elpenor*. *J. Exp. Biol.* 209, 789–800. doi: 10.1242/jeb.02053

Johnson, S. D., and Dafni, A. (1998). Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Funct. Ecol.* 12, 289–297. doi: 10.1046/j.1365-2435.1998.00175.x

Kandori, I., and Ohsaki, N. (1998). Effect of experience on foraging behavior towards artificial nectar guide in the cabbage butterfly, Pieris rapae crucivora (Lepidoptera: Pieridae). *Appl. Entomol. Zool.* 33, 35–42. doi: 10.1303/aez.33.35

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kevan, P. G. (1979). Vegetation and floral colors revealed by ultraviolet light: interpretational difficulties for functional significance. *Am. J. Bot.* 66, 749–751. doi: 10.1002/j.1537-2197.1979.tb06280.x

Kevan, P. G., and Backhaus, W. G. (1998). "Color vision: ecology and evolution in making the best of the photic environment," in *Color Vision: Perspectives From Different Disciplines*, eds G. K. Werner, W. G. Backhaus, and R. Kliegl (New York, NY: deGreuter), 163–183. doi: 10.1515/9783110806984.163

Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? *Trends Plant Sci.* 1:252. doi: 10.1016/1360-1385(96)20008-1

Klomberg, Y., Dywou Kouede, R., Bartoš, M., Mertens, J. E., Tropek, R., Fokam, E. B., et al. (2019). The role of ultraviolet reflectance and pattern in the pollination system of *Hypoxis camerooniana* (Hypoxidaceae). *AoB Plants* 11:lz057.

Knuth, P. (1891). Die Einwirkung der Blütenfarben auf die photographische Platte. *Botanisches Zentralblatt* 48:160.

Koski, M. H. (2020). Macroevolution of flower color patterning: biased transition rates and correlated evolution with flower size. *Front. Plant Sci.* 11:945. doi: 10.3389/fpls.2020.00945

Koski, M. H., and Ashman, T. L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct. Ecol.* 28, 868–877. doi: 10.1111/1365-2435.12242

Koski, M. H., and Ashman, T. L. (2015). An altitudinal cline in UV floral pattern corresponds with a behavioral change of a generalist pollinator assemblage. *Ecology* 96, 3343–3353. doi: 10.1890/15-0242.1

Koski, M. H., and Ashman, T.-L. (2016). Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors. *New Phytol.* 211, 708–718. doi: 10.1111/nph.13921

Koski, M. H., MacQueen, D., and Ashman, T. L. (2020). Floral pigmentation has responded rapidly to global change in ozone and temperature. *Curr. Biol.* 30, 1–7. doi: 10.1017/9781316534588.002

Landi, M., Tattini, M., and Gould, K. S. (2015). Multiple functional roles of anthocyanins in plant-environment interactions. *Environ. Exp. Bot.* 119, 4–17. doi: 10.1016/j.envexpbot.2015.05.012

Lee, D. W., and Gould, K. S. (2002). Why leaves turn red: pigments called anthocyanins probably protect leaves from light damage by direct shielding and by scavenging free radicals. *Am. Sci.* 90, 524–531. doi: 10.1511/2002.39.794

Leonard, A. S., and Papaj, D. R. (2011). 'X' marks the spot: the possible benefits of nectar guides to bees and plants. *Funct. Ecol.* 25, 1293–1301. doi: 10.1111/j.1365-2435.2011.01885.x

Lunau, K. (1991a). 1nnate recognition of flowers by bumblebees (*Bombus terrestris. B. lucorum*; Apidae): optical signals as learning reaction releasers. *Ethology* 88, 203–214. doi: 10.1111/j.1439-0310.1991.tb00275.x

Lunau, K. (1991b). Innate recognition of flowers by bumblebees - orientation of antennae to visual stamen signals. *Can. J. Zool.* 70, 2139–2144. doi: 10.1139/z92-288

Lunau, K. (1992). A new interpretation of flower guide colouration: absorption of ultraviolet light enhances colour saturation. *Plant Syst. Evol.* 183, 51–65. doi: 10.1007/bf00937735

Lunau, K. (2000). The ecology and evolution of visual pollen signals. *Plant Syst. Evol.* 222, 89–111. doi: 10.1007/978-3-7091-6306-1_5

Lunau, K. (2014). Visual ecology of flies with particular reference to colour vision and colour preferences. *J. Comp. Physiol. A* 200, 497–512. doi: 10.1007/s00359-014-0895-1

Lunau, K., Konzmann, S., Winter, L., Kamphausen, V., and Ren, Z. X. (2017). Pollen and stamen mimicry: the alpine flora as a case study. *Arthropod-Plant Inte.* 11, 427–447. doi: 10.1007/s11829-017-9525-5

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K., Ren, Z. X., Fan, X. Q., Trunschke, J., Pyke, G. H., and Wang, H. (2020). Nectar mimicry: a new phenomenon. *Sci. Rep. UK.* 10:7039.

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naive bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 178, 477–489.

Maia, R., Gruson, H., Endler, J. A., and White, T. E. (2019). pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods Ecol. Evol.* 10, 1097–1107. doi: 10.1111/2041-210x.13174

Mangiafico, S. (2020). *rcompanion: Functions to Support Extension Education Program Evaluation. R package version 2.3.25*. Available online at: https://CRAN.R-project.org/package=rcompanion. (accessed March 25, 2020).

Manning, A. (1956). Some aspects of the foraging behaviour of bumble-bees. *Behaviour* 9, 164–200. doi: 10.1163/156853956x00291

Martínez-Harms, J., Hadar, R., Márquez, N., Menzel, R., Shmida, A., Stavenga, D. G., et al. (2020). Enhanced UV-reflection facilitated a shift in the pollination system of the red poppy, *Papaver rhoeas* (Papaveraceae). *Plants* 9:927. doi: 10.3390/plants9080927

McEwen, J. R., and Vamosi, J. C. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proc. R. Soc. B Biol. Sci.* 277, 2957–2965. doi: 10.1098/rspb.2010.0501

Medel, R., Botto-Mahan, C., and Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, Mimulus luteus. *Ecology* 84, 1721–1732. doi: 10.1890/01-0688

Melo, B. T., Mota, T., Schlindwein, C., Antonini, Y., and Oliveira, R. (2018). Floral colour change in *Byrsonima variabilis* (Malpighiaceae) as a visual cue for pollen but not oil foraging by oil-collecting bees. *Sci. Nat. Heidelberg.* 105:46.

Milne, L. J., and Milne, M. (1958). What do animals see? *Am. Schol.* 39–48.

Moyers, B. T., Owens, G. L., Baute, G. J., and Rieseberg, L. H. (2017). The genetic architecture of UV floral patterning in sunflower. *Ann. Bot. London* 120, 39–50. doi: 10.1093/aob/mcx038

Moyroud, E., Wenzel, T., Middleton, R., Rudall, P. J., Banks, H., Reed, A., et al. (2017). Disorder in convergent floral nanostructures enhances signalling to bees. *Nature* 550, 469–474. doi: 10.1038/nature24285

Muchhala, N., Johnsen, S., and Smith, S. D. (2014). Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* 68, 2275–2286.

Müller, B., Glösmann, M., Peichl, L., Knop, G. C., Hagemann, C., and Ammermüller, J. (2009). Bat eyes have ultraviolet-sensitive cone photoreceptors. *PLoS One* 4:e6390. doi: 10.1371/journal.pone.0006390

Nepi, M. (2017). New perspectives in nectar evolution and ecology: simple alimentary reward or a complex multiorganism interaction? *Acta Agrobot* 70:1704.

Nepi, M., Grasso, D. A., and Mancuso, S. (2018). Nectar in plant–insect mutualistic relationships: from food reward to partner manipulation. *Front. Plant Sci.* 9:1063. doi: 10.3389/fpls.2018.01063

Ödeen, A., and Håstad, O. (2010). Pollinating birds differ in spectral sensitivity. *J. Comp. Physiol. A* 196, 91–96. doi: 10.1007/s00359-009-0474-z

Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A., Amaral-Neto, L. P., Sazima, M., et al. (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* 18, 46–55. doi: 10.1111/plb.12322

Papiorek, S., Junker, R. R., and Lunau, K. (2014). Gloss, colour and grip: multifunctional epidermal cell shapes in bee-and bird-pollinated flowers. *PLoS One* 9:e112013. doi: 10.1371/journal.pone.0112013

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: how bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern *phylogenetics* and evolutionary analyses in R. *Bioinformatics* 35, 526–528. doi: 10.1093/bioinformatics/bty633

Peach, K., Liu, J. W., and Mazer, S. J. (2020). Climate predicts UV floral pattern size, anthocyanin concentration, and pollen performance in *Clarkia unguiculata*. *Front. Plant Sci.* 11:847. doi: 10.3389/fpls.2020.00847

Pedersen, T. L. (2020). *patchwork: The Composer of Plots. R package version 1.0.1*. Available online at: https://CRAN.R-project.org/package=patchwork (accessed March 25, 2020).

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/bf00190398

Pélabon, C., Thöne, P., Hansen, T. F., and Armbruster, W. S. (2012). Signal honesty and cost of pollinator rewards in *Dalechampia scandens* (*Euphorbiaceae*). *Ann. Bot. London* 109, 1331–1340. doi: 10.1093/aob/mcs091

Peterson, M. L., Miller, T. J., and Kay, K. M. (2015). An ultraviolet floral polymorphism associated with life history drives pollinator discrimination in *Mimulus guttatus*. *Am. J. Bot.* 102, 396–406. doi: 10.3732/ajb.1400415

Pfadenhauer, J. S., and Klötzli, F. A. (2020). *Global Vegetation: Fundamentals, Ecology and Distribution*. Cham: Springer Nature.

Proctor, M., and Yeo, P. (1973). *The Pollination of Flowers*. London: Collins.

Qian, H., and Jin, Y. (2016). An updated megaphylogeny of plants, a tool for generating plant phylogenies and an analysis of phylogenetic community structure. *J. Plant Ecol.* 9, 233–239. doi: 10.1093/jpe/rtv047

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rae, J. M., and Vamosi, J. C. (2013). Ultraviolet reflectance mediates pollinator visitation in *Mimulus guttatus*. *Plant Spec. Biol.* 28, 177–184. doi: 10.1111/j.1442-1984.2012.00375.x

Ramirez, N., Gil, C., Hokche, O., Seres, A., and Brito, Y. (1990). Biologia floral de una comunidad arbustiva tropical en la Guayana Venezolana. *Ann. Mo. Bot. Gard* 77, 383–397. doi: 10.2307/2399554

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., et al. (2014). A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett* 17, 388–400. doi: 10.1111/ele.12224

Rudis, B. (2020). *hrbrthemes: Additional Themes, Theme Components and Utilities for 'ggplot2'. R Package Version 0.8.0*. Avaialble online at: https://CRAN.R-project.org/package=hrbrthemes (accessed March 25, 2020).

Schlangen, K., Miosic, S., Castro, A., Freudmann, K., Luczkiewicz, M., Vitzthum, F., et al. (2009). Formation of UV-honey guides in *Rudbeckia hirta*. *Phytochemistry* 70, 889–898. doi: 10.1016/j.phytochem.2009.04.017

Scora, R. W. (1964). Dependency of Pollination on Patterns in *Monarda* (Labiatae). *Nature* 204, 1011–1012. doi: 10.1038/2041011a0

Sheehan, H., Moser, M., Klahre, U., Esfeld, K., Dell'Olivo, A., Mandel, T., et al. (2016). MYB-FL controls gain and loss of floral UV absorbance, a key trait affecting pollinator preference and reproductive isolation. *Nat. Genet* 48, 159–166. doi: 10.1038/ng.3462

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *J. Ecol.* 102, 126–135. doi: 10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* 198, 301–310. doi: 10.1111/nph.12135

Skorupski, P., and Chittka, L. (2010). Photoreceptor spectral sensitivity in the bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). *PLoS One* 5:e12049. doi: 10.1371/journal.pone.0012049

Skorupski, P., Döring, T. F., and Chittka, L. (2007). Photoreceptor spectral sensitivity in island and mainland populations of the bumblebee, *Bombus terrestris*. *J. Comp. Physiol. A* 193, 485–494. doi: 10.1007/s00359-006-0206-6

Sponberg, S., Dyhr, J. P., Hall, R. W., and Daniel, T. L. (2015). Luminance-dependent visual processing enables moth flight in low light. *Science* 348, 1245–1248. doi: 10.1126/science.aaa3042

Sprengel, C. K. (1793). *Das Entdeckte Geheimnis der Natur*. Leipzig: Wilhelm Engelmann.

Stavenga, D. G., and Arikawa, K. (2006). Evolution of color and vision of butterflies. *Arthropod Struct. Dev.* 35, 307–318. doi: 10.1016/j.asd.2006.08.011

Théry, M., Pincebourde, S., and Feer, F. (2008). Dusk light environment optimizes visual perception of conspecifics in a crepuscular horned beetle. *Behav. Ecol.* 19, 627–634. doi: 10.1093/beheco/arn024

Thompson, W. R., Meinwald, J., Aneshansley, D., and Eisner, T. (1972). Flavonols: pigments responsible for ultraviolet absorption in nectar guide of flower. *Science* 177, 528–530. doi: 10.1126/science.177.4048.528

Tunes, P., Alves, V. N., Valentin-Silva, A., Batalha, M. A., and Guimarães, E. (2017). Does fire affect the temporal pattern of trophic resource supply to pollinators

and seed-dispersing frugivores in a Brazilian savanna community? *Plant Ecol.* 218, 345–357. doi: 10.1007/s11258-016-0695-5

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signalling. *Ann. Bot. London* 123, 263–276. doi: 10.1093/aob/mcy119

van der Niet, T., and Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol. Evol.* 27, 353–361. doi: 10.1016/j.tree.2012.02.002

Wang, D., Oakley, T., Mower, J., Shimmin, L. C., Yim, S., Honeycutt, R. L., et al. (2004). Molecular evolution of bat color vision genes. *Mol. Biol. Evol.* 21, 295–302. doi: 10.1093/molbev/msh015

White, R. H., Stevenson, R. D., Bennett, R. R., Cutler, D. E., and Haber, W. A. (1994). Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. *Biotropica* 26, 427–435. doi: 10.2307/2389237

Wickham, H., Averick, M., and Bryan, J. (2019). Welcome to the tidyverse. *J. Open Source Softw.* 4:1686. doi: 10.21105/joss.01686

Xu, V., and Plowright, C. (2017). A transferrable change in preferences of floral patterns by bumblebees through reward reversal. *Int. J. Comp. Psychol.* 30:32609.

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The reviewer KL declared a past co-authorship with one of the authors MC to the handling Editor.

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Flower Color Evolution and the Evidence of Pollinator-Mediated Selection

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The evolution of floral traits in animal-pollinated plants involves the interaction between flowers as signal senders and pollinators as signal receivers. Flower colors are very diverse, effect pollinator attraction and flower foraging behavior, and are hypothesized to be shaped through pollinator-mediated selection. However, most of our current understanding of flower color evolution arises from variation between discrete color morphs and completed color shifts accompanying pollinator shifts, while evidence for pollinator-mediated selection on continuous variation in flower colors within populations is still scarce. In this review, we summarize experiments quantifying selection on continuous flower color variation in natural plant populations in the context of pollinator interactions. We found that evidence for significant pollinator-mediated selection is surprisingly limited among existing studies. We propose several possible explanations related to the complexity in the interaction between the colors of flowers and the sensory and cognitive abilities of pollinators as well as pollinator behavioral responses, on the one hand, and the distribution of variation in color phenotypes and fitness, on the other hand. We emphasize currently persisting weaknesses in experimental procedures, and provide some suggestions for how to improve methodology. In conclusion, we encourage future research to bring together plant and animal scientists to jointly forward our understanding of the mechanisms and circumstances of pollinator-mediated selection on flower color.

Keywords: color perception, color preference, flower color variation, pollinator attraction, pollinator behavior, pollinator-mediated selection

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INTRODUCTION

There is an almost bewildering diversity of flower colors and color patterns in flowering plants with colors spanning the entire color spectrum of human and pollinator vision (Menzel and Shmida, 1993), and varying enormously over a range of geographic and temporal scales. Flower color shows, for example, differences at various spatial scales ranging from variation — both continuous and discrete — among individual plants of the same population, plant populations, closely related species, and different flowering communities (e.g., Menzel and Shmida, 1993; Meléndez-Ackerman et al., 1997; Irwin and Strauss, 2005; Ellis and Johnson, 2009; Caruso et al., 2010; **Supplementary Table 1**). Flower color may also show temporal variation within the same individual intrinsically as a result of aging or

extrinsically in response to pollination or changes in abiotic conditions (e.g., Weiss, 1995; Suzuki and Ohashi, 2014; **Supplementary Table 1**).

There is wide agreement that today's diversity of flower colors in angiosperms is largely shaped by variation in the interactions with pollinating animals through the process of natural selection (Schiestl and Johnson, 2013; Van der Niet et al., 2014; Gervasi and Schiestl, 2017). Cumulative evidence for this view arises from the following macro-evolutionary observations (see **Supplementary Table 2** for relevant references): correlations between attributes of flowers and their flower visitors across lineages (i.e., pollination syndromes); pollinator shifts associated with transitions in flower color leading to geographic variation within or across plant lineages or entire plant communities; spatial variation in attraction of pollinators with different color preference resulting in local adaptation; effects of flower color on pollinator behavior causing disassortative mating and reproductive isolation in plant hybrid zones; and the resemblance of color signals of a floral or non-floral model by a mimicking plant (i.e., plant floral mimicry systems).

Pollinators can exert substantial selective pressure on flower color and drive the evolution of flower color signals through preferential visitation and pollination efficiency, because animal pollination involves an interaction between the various plant and floral attributes of flower color signaling on the one side, and the sensory abilities and behavioral responses of the potential pollinators on the other (Chittka and Menzel, 1992; Menzel and Shmida, 1993; Chittka and Raine, 2006). Variation in flower perception, detection and preferences by pollinators likely results in variable visitation, pollination success and male and/or female fitness among color phenotypes (e.g., Waser and Price, 1981; Campbell et al., 1997). Thus, the way pollinators perceive and discriminate differently colored flowers, and how they respond to the perceived differences through preferential visitation, may drive flower color divergence among morphs, populations or species (Campbell et al., 1997). Similarly, how pollinators perceive and respond to variation in flower color within populations should determine the target, shape and strength of pollinator-mediated selection (Waser and Price, 1981).

Demonstrating pollinators as agents of selection in natural populations is important because it can provide the missing linkage between variation in animal vision and color preferences, and macro-evolutionary patterns of variation in flower color in angiosperms. However, while much work has been done to document and explain variation in flower color among morphs within color di- or polymorphic populations, and among populations and species, quantification of continuous color variation within populations and possible selection on it is limited (Rausher, 2008; Sapir et al., 2021). Few studies have measured the form and strength of natural selection on flower color, and those that estimate the importance of pollinators for selection are rare (**Table 1**).

Pollinators can be expected to exert directional selection on flower color to increase detectability and stabilizing selection to increase pollinator constancy (Waser and Price, 1981; Chittka, 1997; Chittka et al., 1997). This is because, in natural populations, a flower color signal should serve two functions: First, it should contrast against the background for detectability by foraging pollinators (Giurfa et al., 1996; Menzel et al., 1997; Koski, 2020), and second, it should contrast against flowers of species co-occurring within the same community to ensure pollinator constancy and conspecific pollen transfer (Chittka and Menzel, 1992; Menzel and Shmida, 1993; Chittka, 1997; Chittka et al., 1997). Furthermore, because pollinators may choose flowers based on a set of phenotypic characters including, for example, inflorescence height and display size, flower color may also be subject to correlational selection favoring a combination of flower color with other pollinator attractive characters.

The form and strength of phenotypic selection in natural populations is typically quantified by regression analysis of relative fitness against standardized quantitative trait values across a large sample of individuals exhibiting substantial phenotypic variation (Price, 1970; Arnold and Wade, 1984). Here, the estimated equation takes the form of directional selection where the regression coefficient S (i.e., the selection differential in univariate analysis) or β_i (i.e., the selection gradient in multivariate models) is significantly < 0 indicating negative directional selection or significantly > 0 indicative for positive directional selection (Lande and Arnold, 1983). While selection differentials depict the total selection acting on a character, estimates of selection gradients control for possible covariances between correlated characters and therefore determine the direct targets of selection (Lande and Arnold, 1983). Non-linear selection is detected through second-order polynomial regression, whereby stabilizing selection occurs when the quadratic regression coefficient γ_i is significantly < 0, and disruptive selection when $\gamma_i > 0$ (Lande and Arnold, 1983; Stinchcombe et al., 2008). Furthermore, selection may favor phenotypic integration of two or more traits when selection gradients of trait combinations γ_{ij} are significantly different from 0 (Lande and Arnold, 1983; Phillips and Arnold, 1989).

In the context of pollination, the causes of selection (i.e., pollinators as selective agents) can be identified by either experimentation or modeling. For example, experimenting may be through manipulation of the pollination environment (i.e., a hand-pollination treatment to remove variation in fitness that is not associated with variation in the interaction with pollinators; Caruso et al., 2018; Sletvold, 2019). On the other hand, statistical estimation approaches may be used with structural equation modeling estimation (i.e., estimating the causal relationship of pollinator interactions with plant fitness; Souto-Vilarósa et al., 2018; Rodriguez-Castañeda et al., 2020; Brunet et al., 2021).

In this review, we aim to summarize and discuss existing studies that have quantified selection on continuous flower color variation, and the evidence of pollinators as potential selective agents. In the first part, we synthesize the results of selection experiments in natural or experimental populations displaying continuous flower color variation. In the second part, we discuss the evidence for pollinator-mediated selection in light of characterization of flower color attributes and their variation, followed by pollinator visual and cognitive processing of the obtained signal (perception and detection of specific flower color attributes), the consequences for pollinator foraging behavior, and the context-dependence of selection (**Figure 1**). We

TABLE 1 | Summary of estimates of selection on continuous flower color variation in published studies and the proportion of significant estimates shown in brackets.

Color Trait	*S*	β	γ_{ii}	γ_{ij}	Poll
Overall	6 (3)	18 (7)	10 (3)	6 (3)	12 (7)
Pigment concentration	1 (1)	4 (2)	3 (2)	1 (0)	3 (1)
Corolla color	2 (2)	6 (1)	2 (0)	2 (0)	2 (1)
Hue	1 (0)	5 (2)	3 (1)	2 (1)	4 (2)
Brightness	1 (0)	5 (2)	3 (1)	2 (0)	4 (2)
Saturation	1 (0)	4 (0)	2 (0)	1 (1)	3 (1)
Patterns/Nectar guides	2 (0)	4 (1)	3 (1)	1 (0)	3 (2)
UV pattern	0	2 (2)	1 (0)	1 (0)	2 (1)

Poll corresponds to any study that determined pollinators as agent of selection through modeling of pollinator visitation or experimentation using a hand-pollination treatment. S, selection differential; β, directional selection gradient; γ_{ii}, quadratic selection gradient; γ_{ij}, correlational selection gradient.

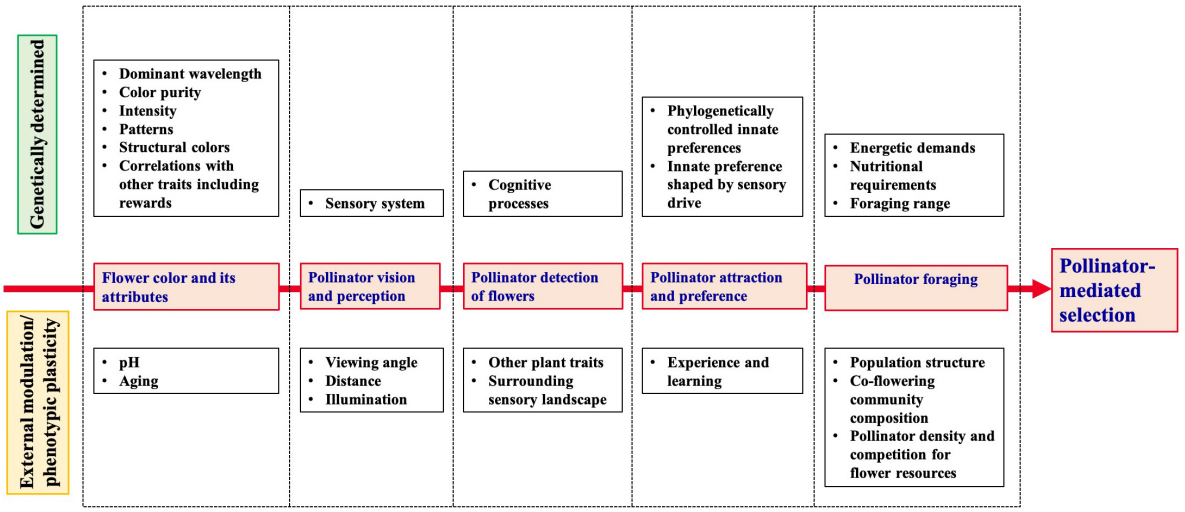


FIGURE 1 | Schematic overview of the successive ways in the interaction of plants and pollinators involving flower colors, and how they impact on the outcome of pollinator-mediated selection on flower color.

highlight the gaps in knowledge and problems in experimental procedures, and provide suggestions for future directions. Here, we raise some of the unresolved questions to forward the understanding of how the interaction with pollinating animals may drive and maintain variation in flower color within and among natural populations.

REVIEW

In recent years, a number of studies have experimentally investigated the shape, strength and context-dependence of pollinator-mediated selection on flowering traits that mediate the interaction with pollinators in natural populations (reviewed in Caruso et al., 2018; Sletvold, 2019). Yet, we are aware of only 18 studies that aimed to quantify selection on continuous variation in petal coloration in the context of pollination (**Tables 1**, **2**). Moreover, of these studies, only six used a hand-pollination treatment to estimate selection gradients associated with pollinator interactions (Caruso et al., 2010; Parachnowitsch and Kessler, 2010; Lavi and Sapir, 2015; Sletvold et al., 2016; Zhang et al., 2017; Souto-Vilarósa et al., 2018; **Supplementary Table 3**). Some other studies used modeling approaches linking the flower color phenotype - fitness relationship to pollinator visitation data to determine the contribution of pollinators to observed total selection (e.g., Veiga et al., 2015; Rodriguez-Castañeda et al., 2020; Brunet et al., 2021; **Supplementary Table 3**).

It is striking that studies have rarely detected significant evidence for directional net selection (which includes all possible causes of selection) on achromatic color parameters. In two studies, natural selection was found to act in a linear manner favoring less bright flowers in *Lobelia siphilitica* in one of these studies (Caruso et al., 2010), and brighter and contrast-rich petal colorization in the deceptive orchid *Anacamptis morio* in the other (Sletvold et al., 2016). In addition, the study by Sletvold et al. (2016) confirmed that pollinators accounted for 100% of observed net selection among open-pollinated plants. In addition, Brunet et al. (2021) found, in a population of *Medicago sativa*, that bumblebees (*Bombus impatiens*) preferred darker flowers but this was due to correlational selection with flower number. That bumblebees use achromatic flower color information (i.e., green contrast) and

preferentially chose flowers with increased brightness resulting in significant directional selection, when modeled through the bees visual system, was also demonstrated by Renoult et al. (2013) in an experimental greenhouse setup, where bumblebees were freely foraging on potted cornflower, *Centaurea cyanus*.

Results of these studies contrast with the well-established finding from behavioral experiments that bees choose colors on the basis of chromatic signals and not their brightness (Ng et al., 2020). The above studies, that indicate the importance of color brightness for pollinator choice and foraging were all carried out in the wild or in large artificial populations. Laboratory studies, on the other hand, have indicated the importance of color purity (Lunau, 1990; Rohde et al., 2013). This inconsistency between laboratory behavioral tests and the behavior of wild bees awaits explanation (Ng et al., 2020).

There are few evidences for pollinators selecting on chromatic flower color traits (**Table 2**). In *Gentiana lutea*, which varies in flower color from yellow to orange, Veiga et al. (2015) found selection for increased yellowness as yellow flowers received higher pollinator visitation than flowers of alternative colors. In the study of *Medicago sativa* by Brunet et al. (2021) alfalfa leafcutting bees, *Megachile rotundata*, exerted stabilizing selection on hue, but neither *Apis mellifera* nor *Bombus impatiens* showed preferences for flower hue resulting in non-significant selection when summed over all bees.

Some studies have identified significant selection on achromatic or chromatic color signals, that was not clearly linked to the interactions with pollinators (e.g., Wassink and Caruso, 2013; Lavi and Sapir, 2015; Veiga et al., 2015; Souto-Vilarósa et al., 2018; **Table 2**). In *Gentiana lutea*, for example, flower yellowness significantly influenced both pollinator visitation and escape from seed predators, which resulted in significant net selection on flower color (Veiga et al., 2015). Wassink and Caruso (2013) detected significant net selection on saturation in *Lobelia siphilitica,* but only in hermaphrodite plants and only in the presence of co-flowering *Mimulus ringens*. In a population of *Iris pumilla*, Souto-Vilarósa et al. (2018) detected significant selection for increased anthocyanin concentration in the blue-flowered form, but no selection was detected in the purple morph. In an among-population study of selection in dimorphic *Iris lutescens*, Souto-Vilarósa et al. (2018) found some evidence for directional selection for increased flavonoid concentration in a yellow-flowered morph, and for decreased anthocyanin concentration consistent across the yellow- and purple-flowered morph. However, this was not a consistent pattern across other populations in which the two morphs co-occur.

Non-pollinator-mediated linear directional selection, as determined by experiment through a hand-pollination treatment, has been found in some studies (Caruso et al., 2010; Lavi and Sapir, 2015; Sletvold et al., 2016; Souto-Vilarósa et al., 2018; **Table 2**). Both Caruso et al. (2010) and Sletvold et al. (2016) detected selection on flower brightness among plants experimentally supplemented with pollen. Lavi and Sapir (2015) and Souto-Vilarósa et al. (2018) found selection, which was non-pollinator-mediated, for increased anthocyanin pigment concentration in *Iris atropurpurea* in one year of study and in *I. lutescens*, respectively. Further, Souto-Vilarósa et al. (2018) also found non-pollinator-mediated selection that was positive for flavonoid concentration in the yellow morph of *I. lutescens* and negative for the purple morph, suggesting disruptive selection within dimorphic populations.

Some studies have also found evidence for significant quadratic selection on flower color traits (**Table 3**). For example, in *Mimulus luteus* selection on nectar guide shape variables through pollinator visitation was disruptive (Medel et al., 2003), but it is unclear whether this translates into net selection through seed production. Both Lavi and Sapir (2015) and Souto-Vilarósa et al. (2018) found significant stabilizing selection on anthocyanin pigment concentration in *Iris atropurpurea* and the blue-flowered morph of *I. pumilla*, respectively, but selection was significantly associated with pollinator interactions only in the latter case.

Evidence for correlational selection among flower color traits and other traits involved in pollinator interactions is generally scarce (**Table 1**). Few studies have estimated selection acting on combinations of different color attributes, or the integration of flower color with flower morphology, and no study has investigated a possible flower color - flower scent association as target of selection (**Table 4**). Among the four studies quantifying correlational selection (Medel et al., 2003; Campbell et al., 2012; Renoult et al., 2013; Brunet et al., 2021), three included flower size - flower color associations, two additionally included plant height - flower color associations, and one measured correlational selection on flower number and flower color. Brunet et al. (2021) detected significant selection for more flowers of higher saturation and reduced brightness by bumblebees in *Medicago sativa* but selection summed over all bees was only significant involving color saturation. And the study by Renoult et al. (2013) found selection for larger flowers of increased blue hue in *Centaurea cyanus*. This indicates that trait combinations involving flower color are important in at least some instances. However, most studies report weak correlations among flower color phenotype and flowering morphology, and consequently did not estimate correlational selection.

In synthesis, it appears that pollinator-mediated selection on flower color is expected to influence its evolution, but is difficult to observe and quantify in natural populations. This difficulty arises, we suggest, because of several aspects in the interplay between plant color phenotypes and pollinator sensory ecology and foraging behavior. These include the complexity of flower color parameters to which pollinators can respond, mechanisms of animal color vision and behavioral flexibility as well as the environmental context (**Figure 1**).

Regarding pollinators, we suggest that the following factors may influence flexibility of pollinator preference expression for flower color when foraging in wild populations and weaken expected pollinator-mediated selection: (a) a prevalent unjustified assumption of innate fixed sensory capacities and preferences versus individual and learned acquisition; (b) poorly understood mechanisms and outcomes of flower color perception, discrimination and behavior and their interactions with the biotic and abiotic environment; and (c) an insufficient consideration of how pollinator visitation to plants and flowers is influenced by the likelihood of pollinator choices before any color preferences might come into play.

TABLE 2 | Summary of linear phenotypic selection gradients (± SE) extracted from the literature for net selection (β_C), non-pollinator-mediated selection (β_{HP}) and pollinator-mediated selection ($\Delta\beta_{Poll}$) on flower color estimated in natural plant populations and experimental populations.

Species	Pollinator	Trait	β_C	β_{HP}	$\Delta\beta_{Poll}$	Reference
Natural populations						
I.tenuituba, I. aggregata	Hummingbirds, hawkmoths	Optical density	na	na	0.01 ± 0.12	Campbell et al. (1997)*
Mimulus luteus	Insects, hummingbirds	Guide shape CVA1	na	na	0.011 ± 0.06	Medel et al. (2003)*
Mimulus luteus	Insects, hummingbirds	Guide shape CVA2	na	na	−0.007 ± 0.06	
Claytonia virginica	Solitary bees	Corolla color	0.019	na	na	Frey (2004)
W. albomarginata	Solitary bees	R440/R530	na	na	0.179 ± 0.45	Campbell et al. (2012)*
W.albomarginata	Solitary bees	PC1 (Color hexagon distance)	na	na	−0.060 ± 0.44	
Iris atropurpurea, Year 1	Eucera bees	Anthocyanin conc.	0.203 ± 0.15	0.149 ± 0.17	0.054	Lavi and Sapir (2015)
Iris atropurpurea, Year 2	Eucera bees	Anthocyanin conc.	0.116 ± 0.09	**0.173 ± 0.09**	−0.057	
Iris haynei, Year 1	Eucera bees	Anthocyanin conc.	0.104 ± 0.13	0.081 ± 0.12	0.023	
Iris haynei, Year 2	Eucera bees	Anthocyanin conc.	0.087 ± 0.11	0.102 ± 0.11	−0.015	
Gentiana lutea	Bumblebees	PC1 (Yellowness)	**0.238 ± 0.08**	na	na	Veiga et al. (2015)
Anacamptis morio	Bumblebee queens	Brightness	0.28 ± 0.17	**−0.130 ± 0.04**	**0.42**	Sletvold et al. (2016)
Anacamptis morio	Bumblebee queens	Lip patch size	−0.25 ± 0.15	0.077 ± 0.04	−0.33	
Anacamptis morio	Bumblebee queens	Lip patch contrast	**0.50 ± 0.17**	−0.017 ± 0.04	**0.51**	
Anacamptis morio	Bumblebee queens	Lip spot area	−0.12 ± 0.14	0.018 ± 0.04	−0.14	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.056 ± 0.12	na	na	Gross et al. (2016)
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.077 ± 0.08	na	na	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.027 ± 0.06	na	na	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.051 ± 0.05	na	na	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	0.021 ± 0.07	na	na	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.085 ± 0.04	na	na	
Gymnadenia conopsea	Butterflies, flies	PC7 (Corolla color)	−0.090 ± 0.06	na	na	
Caltha scaposa	Bees, flies	UV bulls-eye size	−0.08 ± 0.06	**6.05 ± 0.88**	−6.13	Zhang et al. (2017)
Caltha scaposa	Bees, flies	UV proportion	0.14 ± 0.06	**−4.63 ± 0.68**	4.77	
Iris lutescens	Apoid bees	Anthocyanin conc.	**−0.19 ± 0.002**	n.s.	n.s.	Souto-Vilarósa et al. (2018)
Iris lutescens	Apoid bees	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris lutescens, yellow	Apidae	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris lutescens, yellow	Apidae	Flavonoid conc.	**0.35 ± 0.01**	**na**	n.s.	
Iris lutescens, purple	Apidae	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris lutescens, purple	Apidae	Flavonoid conc.	**−0.20 ± 0.01**	**na**	n.s.	
Iris pumila, purple	Apidae	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris pumila, blue	Apidae	Anthocyanin conc.	**−0.25 ± 0.06**	**0.42± 0.003**	**−0.67**	
Iris pumila, yellow	Apidae	Anthocyanin conc.	na	na	na	
Iris pumila, purple	Apidae	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris pumila, blue	Apidae	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris pumila, yellow	Apidae	Flavonoid conc.	na	na	na	
A.coriophora coriophora	Bees	PC1 (RGB values)	0.002 ± 0.04	na	na	Joffard et al. (2020)
A.coriophora coriophora	Bees	PC1 (RGB values)	0.08 ± 0.12	na	na	
A.coriophora coriophora	Bees	PC1 (RGB values)	−0.02 ± 0.05	na	na	
A.coriophora fragrans	Bees	PC1 (RGB values)	−0.02 ± 0.06	na	na	
A.coriophora fragrans	Bees	PC1 (RGB values)	0.02 ± 0.05	na	na	
A.coriophora martrinii	Bees	PC1 (RGB values)	−0.04 ± 0.08	na	na	
Silene littorea	Bees, butterflies, *Hadena*	Anthocyanin conc.	−0.11 ± 0.10	na	na	Rodriguez-Castañeda et al. (2020)
Silene littorea	Bees, butterflies, *Hadena*	Corolla color	−0.1 ± 0.17	na	na	
Medicago sativa	Bees	Brightness	n.s.	na	na	Brunet et al. (2021)

(Continued)

TABLE 2 | Continued

Species	Pollinator	Trait	β_C	β_{HP}	$\Delta\beta_{Poll}$	References
Medicago sativa	Bees	Saturation	n.s.	na	na	
Medicago sativa	Bees	Hue	n.s.	na	na	
Experimental populations						
I.tenuituba, I. aggregata	Hummingbirds, hawkmoths	Optical density	na	na	**0.19 ± 0.05**	Campbell et al. (1997)*
Lobelia siphilitica	Bumblebees	Brightness	−0.050 ± 0.06	**0.111 ± 0.05**	**−0.161**	Caruso et al. (2010)
Lobelia siphilitica	Bumblebees	Saturation	−0.019 ± 0.06	0.074 ± 0.05	−0.093	
Lobelia siphilitica	Bumblebees	Hue	−0.019 ± 0.06	**0.101 ± 0.05**	−0.120	
Penstemon digitalis	Bumblebees	Nectar guide	−0.003 ± 0.01	−0.005 ± 0.01	0.002	Parachnowitsch and Kessler (2010)
Penstemon digitalis	Bumblebees	Nectar guide	−0.02± 0.03	na	na	Parachnowitsch et al. (2012)
Lobelia siphilitica, H	Bumblebees	Brightness	−0.062 ± 0.04	na	na	Wassink and Caruso (2013)
Lobelia siphilitica, H	Bumblebees	Saturation	−0.017 ± 0.04	na	na	
Lobelia siphilitica, H	Bumblebees	Hue	0.062 ± 0.04	na	na	
Lobelia siphilitica, F	Bumblebees	Brightness	−0.015 ± 0.03	na	na	
Lobelia siphilitica, F	Bumblebees	Saturation	0.115 ± 0.04	na	na	
Lobelia siphilitica, F	Bumblebees	Hue	−0.012 ± 0.03	na	na	
Centaurea cyanus	Bumblebees	PC1 (Brightness)	0.007	na	na	Renoult et al. (2013)
Centaurea cyanus	Bumblebees	PC2 (Blue-violet)	0.029	na	na	
Centaurea cyanus	Bumblebees	PC3 (UV)	**−0.061**	na	na	

*Significant selection gradients at $P < 0.05$ are highlighted in bold. All selection gradients were estimated using multiple regression following the Lande and Arnold (1983) protocol. Multiple rows of the same species for the same trait correspond to different populations if not otherwise indicated. *Note that Campbell et al. (1997), Campbell et al. (2012), and Medel et al. (2003) estimated selection gradients based on pollinator visitations as fitness response rather than measurements of components of female fitness. I, Ipomopsis; W, Wahlenbergia; A, Anacamptis.; F, Female; H, Hermaphrodite.*

With respect to floral phenotypes, we similarly consider that the following diminish detectability of selection: (a) an insufficient quantification and discrimination of relevant aspects of flower color and color patterning; (b) a lack of considering functional relationships between flower color and other floral traits including the possible association with a floral resource such as nectar or pollen and their effects on pollinator foraging; (c) a poorly understood role of abiotic factors for determining variation in color among and within plant individuals.

In the following sections, we shall discuss these challenges arising in the context of animals foraging for floral resources involving flower color and attributes of the plants and flowers they visit.

Flower Color Attributes, Its Variation and Function

The color appearance of a flower is determined by a complex interaction of chemical, physical and morphological factors on the one hand, and physiological and neural parameters on the other hand. A color phenotype as perceived by a flower visitor is predominantly characterized by its hue (the dominant spectral descriptor), saturation (the spectral purity) and brightness (the intensity of spectral reflectance), and also by the contrast with surrounding color (Bukovac et al., 2017; van der Kooi et al., 2019; **Figure 2**). Differences in colors among and within flowers are modulated through variation in the identity and concentration of plant pigments in distinct cell layers of the petals or other signaling organs (most commonly anthocyanins, flavonoids and carotenoids; van der Kooi et al., 2016), co-pigmentation (Yabuya et al., 2000; Mizuno et al., 2015) and epidermis cell shape (Kraaij and van der Kooi, 2019; Stavenga et al., 2020). In addition, structural colors (gloss, polarization, iridescence) and fluorescence can influence the appearance of flowers (Vignolini et al., 2013; van der Kooi et al., 2016). Iridescence, for example, can be considered to corrupt color identity, because the perceived color is dependent on the viewing angle (Kjernsmo et al., 2018). Similarly, fluorescence as often possessed by pollen (Mori et al., 2018) may also alter the perception of flower color, because pollen may thus absorb light in a distinct range of wavelength and reflect light in another range of wavelength, causing a bathochromic shift.

All such color components may have important signaling cues for pollinators (Lunau, 2000; Garcia et al., 2014; Horth et al., 2014; Verhoeven et al., 2018; van der Kooi et al., 2019). For example, colored pan traps used as artificial flower mimics often catch more flower-visitors if fluorescent colors are displayed (Shrestha et al., 2019), suggesting their potential role in pollinator attraction. The functional significance of structural colors as a signaling cue for pollinators has been shown in behavioral assays under laboratory conditions (Papiorek et al., 2014; van der Kooi et al., 2019). Yet, their importance for wild pollinators freely foraging in natural plant populations remains to be explored

TABLE 3 | Summary of quadratic selection gradients (± SE) extracted from the literature for net selection (γ_C), non-pollinator-mediated selection (γ_{HP}) and pollinator-mediated selection ($\Delta\gamma_{Poll}$) on flower color estimated in natural plant populations and experimental populations.

Species	Trait	γ_C	γ_{HP}	γ_{Poll}	Reference
Natural populations					
I. tenuituba, I. aggregata	Optical density	na	na	0.03 ± 0.10	Campbell et al. (1997)*
Mimulus luteus	Guide shape CVA1	na	na	**6.338 ± 1.38**	Medel et al. (2003)*
Mimulus luteus	Guide shape CVA2	na	na	**2.580 ± 0.75**	
Claytonia virginica	Corolla color	−0.009	na	na	Frey (2004)
W. albomarginata	R440/R530	na	na	−0.091 ± 0.42	Campbell et al. (2012)*
W. albomarginata	PC1 (Color hexagon distance)	na	na	−0.563 ± 0.45	
Iris atropurpurea, Year 1	Anthocyanin conc.	−0.958 ± 1.99	0.018 ± 2.35	−0.976	Lavi and Sapir (2015)
Iris atropurpurea, Year 2	Anthocyanin conc.	1.121 ± 0.59	**1.329 ± 0.73**	−0.208	
Iris haynei, Year 1	Anthocyanin conc.	1.946 ± 2.43	2.266 ± 1.71	−0.320	
Iris haynei, Year 2	Anthocyanin conc.	−0.060 ± 1.04	−0.554 ± 0.99	0.494	
Anacamptis morio	Brightness	−0.270 ± 0.25	0.004 ± 0.06	−0.270	Sletvold et al. (2016)
Anacamptis morio	Lip patch size	0.320 ± 0.17	−0.022 ± 0.06	0.340	
Anacamptis morio	Lip patch contrast	−0.022 ± 0.26	−0.021 ± 0.06	−0.001	
Anacamptis morio	Lip spot area	0.074 ± 0.21	−0.002 ± 0.05	0.076	
Iris lutescens, yellow	Anthocyanin conc.	n.s.	n.s.	n.s.	Souto-Vilarósa et al. (2018)
Iris lutescens, yellow	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris lutescens, purple	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris lutescens, purple	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris lutescens, yellow	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris lutescens, yellow	Flavonoid conc.	n.s.	na	n.s.	
Iris lutescens, purple	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris lutescens, purple	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris pumila, purple	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris pumila, blue	Anthocyanin conc.	n.s.	n.s.	n.s.	
Iris pumila, purple	Flavonoid conc.	n.s.	n.s.	n.s.	
Iris pumila, blue	Flavonoid conc.	**−0.900 ± 0.12**	0.003 ± 0.03	**−0.903**	
Medicago sativa	Brightness	n.s.	na	na	Brunet et al. (2021)
Medicago sativa	Saturation	n.s.	na	na	
Medicago sativa	Hue	n.s.	na	na	
Experimental populations					
Penstemon digitalis	Nectar guide	0.004 ± 0.01	**−0.006 ± 0.01**	0.01	Parachnowitsch and Kessler (2010)
Centaurea cyanus	PC1 (Brightness)	0.020	na	na	Renoult et al. (2013)
Centaurea cyanus	PC2 (Blue-violet)	0.030	na	na	
Centaurea cyanus	PC3 (UV)	0.003	na	na	

*Significant selection gradients at $P < 0.05$ are highlighted in bold. All selection gradients were estimated using multiple regression following the Lande and Arnold (1983) protocol. *Note that Campbell et al. (1997), Campbell et al. (2012), and Medel et al. (2003) estimated selection gradients based on pollinator visitations as fitness response rather than measurements of components of female fitness. I, Ipomopsis; W, Wahlenbergia; A, Anacamptis.*

(Iriel and Lagorio, 2010; Garcia et al., 2019; Lunau et al., 2020). Further, trait manipulation experiments have demonstrated that masking of flower nectar guides significantly reduced pollinator visits (Hansen et al., 2012). However, details of color patterning as potential target of selection by pollinators have rarely been evaluated (but see Medel et al., 2003; Parachnowitsch and Kessler, 2010; Parachnowitsch et al., 2012; Sletvold et al., 2016; Zhang et al., 2017). That both Medel et al. (2003) and Sletvold et al. (2016) found significant selection on traits related to color patterning within flowers (**Tables 2**, **3**) supports the view of their relevance for pollinator foraging decisions or pollination efficiency, and therefore may be more important than primary hue for pollinators selecting on flower color.

In previous studies, variation in flower colors have been measured in the following ways, each with advantages and disadvantages: by comparison of flower petals to color charts (three studies), digital photography (five), pigment extraction and spectrometry of extracts (three) or using direct spectral photometry of floral tissues in either laboratory or field (seven; **Supplementary Table 3**). With the exception of digital

TABLE 4 | Summary of correlational selection gradients (± SE) extracted from the literature for net selection (γ_C), non-pollinator-mediated selection (γ_{HP}) and pollinator-mediated selection ($\Delta\gamma_{Poll}$) on flower color estimated in natural plant populations and experimental populations.

Species	Trait combination including flower color	γ_C	γ_{HP}	γ_{Poll}	Reference
Natural populations					
Wahlenbergia albomarginata	R440/R530 × flower size	na	na	0.320 ± 0.35	Campbell et al. (2012)*
Wahlenbergia albomarginata	PC1 (Color hexagon distance) × flower size	na	na	0.013 ± 0.49	
Mimulus luteus	Guide shape CVA1 × plant height	na	na	0.058 ± 0.28	Medel et al. (2003)*
Mimulus luteus	Guide shape CVA1 × corolla size	na	na	−0.210 ± 0.30	
Mimulus luteus	Guide shape CVA1 × guide shape CVA2	na	na	0.187 ± 0.25	
Mimulus luteus	Guide shape CVA2 × plant height	na	na	0.226 ± 0.32	
Mimulus luteus	Guide shape CVA2 × corolla size	na	na	0.073 ± 0.30	
Mimulus luteus	Guide shape CVA2 × plant height	na	na	−0.564 ± 0.34	
Mimulus luteus	Guide shape CVA2 × corolla size	na	na	−0.332 ± 1.40	
Medicago sativa	Brightness × flowers per raceme	n.s.	na	na	Brunet et al. (2021)
Medicago sativa	Saturation × flowers per raceme	**0.370**	na	na	
Medicago sativa	Hue × flowers per raceme	n.s.	na	na	
Centaurea cyanus	PC1 (Brightness) × PC2 (Blue-violet)	0.004	na	na	Renoult et al. (2013)
Centaurea cyanus	PC1 (Brightness) × PC3 (UV)	−0.044	na	na	
Centaurea cyanus	PC1 (Brightness) × flower size	−0.021	na	na	
Centaurea cyanus	PC1 (Brightness) × plant height	−0.017	na	na	
Centaurea cyanus	PC2 (Blue-violet) × PC3 (UV)	−0.016	na	na	
Centaurea cyanus	PC2 (Blue-violet) × flower size	**0.101**	na	na	
Centaurea cyanus	PC2 (Blue-violet) × plant height	−0.038	na	na	
Centaurea cyanus	PC3 (UV) × flower size	−0.018	na	na	
Centaurea cyanus	PC3 (UV) × plant height	0.028	na	na	

*Significant selection gradients at $P < 0.05$ are highlighted in bold. All selection gradients were estimated using multiple regression following the Lande and Arnold (1983) protocol. *Note that Campbell et al. (1997) and Medel et al. (2003) estimated selection gradients based on pollinator visitations as fitness response rather than measurements of components of female fitness.*

photography, a common limitation is that these methods offer little opportunity to separate and study details of flower color components such as the above described: patterning, transitions or contrasts within petals; nectar guides; anther and pollen colors; as well as aspects of polarization, florescence and shine. In addition, it may also be that some methods such as multiple color categories do not provide a sufficiently fine resolution to describe color variation in the analysis of selection. And even for commonly used spectrometry it may be difficult to reliably detect subtle differences in reflectance among individuals since even repeated measurements of the same flower can result in some deviances (Garcia et al., 2014; Johnsen, 2016).

It is important to separate different components of flower-visitor-subjective color appearance of flowers (e.g., hue, saturation, brightness, and other potentially visible parameters) for appropriately defining potential targets of selection, as evident from laboratory experiments. For example, bees presented with two yellow, less saturated colors and two blue, more saturated colors, preferentially chose the more saturated colors (i.e., higher spectral purity) regardless of the primary hue (yellow vs. blue; Papiorek et al., 2013; Rohde et al., 2013). Moreover, the few studies that have analyzed selection by separating hue, saturation and brightness, all suggest significant direct selection on one or multiple of such components (**Tables 2**, **3**).

Various extrinsic factors can affect the appearance of colors and can thus influence pollinator visual perception of flowers, and consequently relationships between flower color and plant fitness. For example, many pollinators are active over a range of daylight and weather conditions (Corbet et al., 1993; Lawson and Rands, 2019), and may therefore experience temporal variation in illumination blurring intrinsic flower color identity based on pigmentation, which influences pollinator foraging (Arnold and Chittka, 2012). Furthermore, plants are exposed to spatial and temporal variation in microhabitats (e.g., direct sunlight or shade, darker or lighter environmental context), and so may display variation in color appearance or contrast with background independent of pigmentation (Arnold and Chittka, 2012). In addition, some plants exhibit intra-individual color changes (Weiss, 1995; Ohashi et al., 2015). To understand the impacts of the visual sensory context on pollinator-mediated selection requires to determine how such modulations in flower color perception may influence pollinator responses and the relationship of flower color and plant fitness.

Recent advances in digital photography and analytical software provide promising new avenues to characterize flower coloration in more detail (Garcia et al., 2014; Verhoeven et al., 2018). For example, Verhoeven et al. (2018), developed a false color photography technique, that combines digital image layers of flowers to visualize structural colors and access flower color

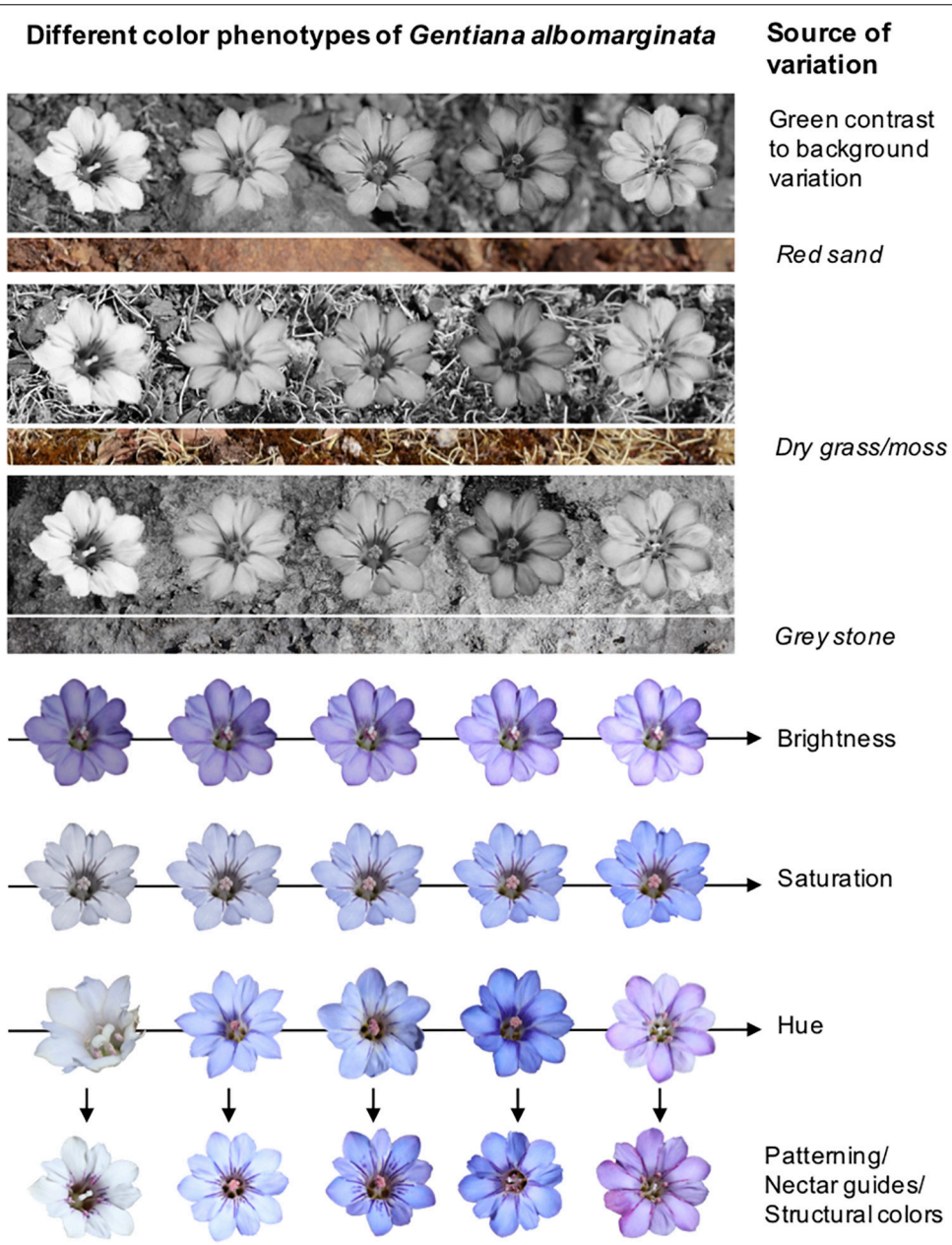


FIGURE 2 | Representation of possible sources of variation in the color appearance of flowers exemplified in the alpine herb *Gentiana albomarginata* as a potential pollinator approaches flowers from far to near. Upper panels: Variation in green contrast between differently colored flowers and different backgrounds as a result of longwave photoreceptor perception. Lower panels: Variation in hue, saturation and brightness, which can be further modulated by variation in petal color patterning and nectar guides, or by variation in structural colors as here the presence and absence of fluorescent purple-colored pollen presented in male but not in female phase flowers.

variation as perceived by pollinators (**Figures 3**, **4**). The use of this method recently allowed Lunau et al. (2020) to quantify shine across a large taxonomic sample of flowers, and the same procedure can also easily be applied to access within-population variation. Also, Hsu et al. (2018) used digital photography and sophisticated algorithms for faster digital image processing to quantify petal color gradients and spot patterns in *Sinningia speciosa*.

A further advantage of digital photography is the possibility to consider the color contrast between flower signal and the background. For example, integration of background color into the analysis of individual color variation has been successfully applied in areas of animal research such as habitat adaptation of body color in lizard (Stevens et al., 2007; Tong et al., 2019). This suggests that its application in the study of flower colors may also provide new insights for the

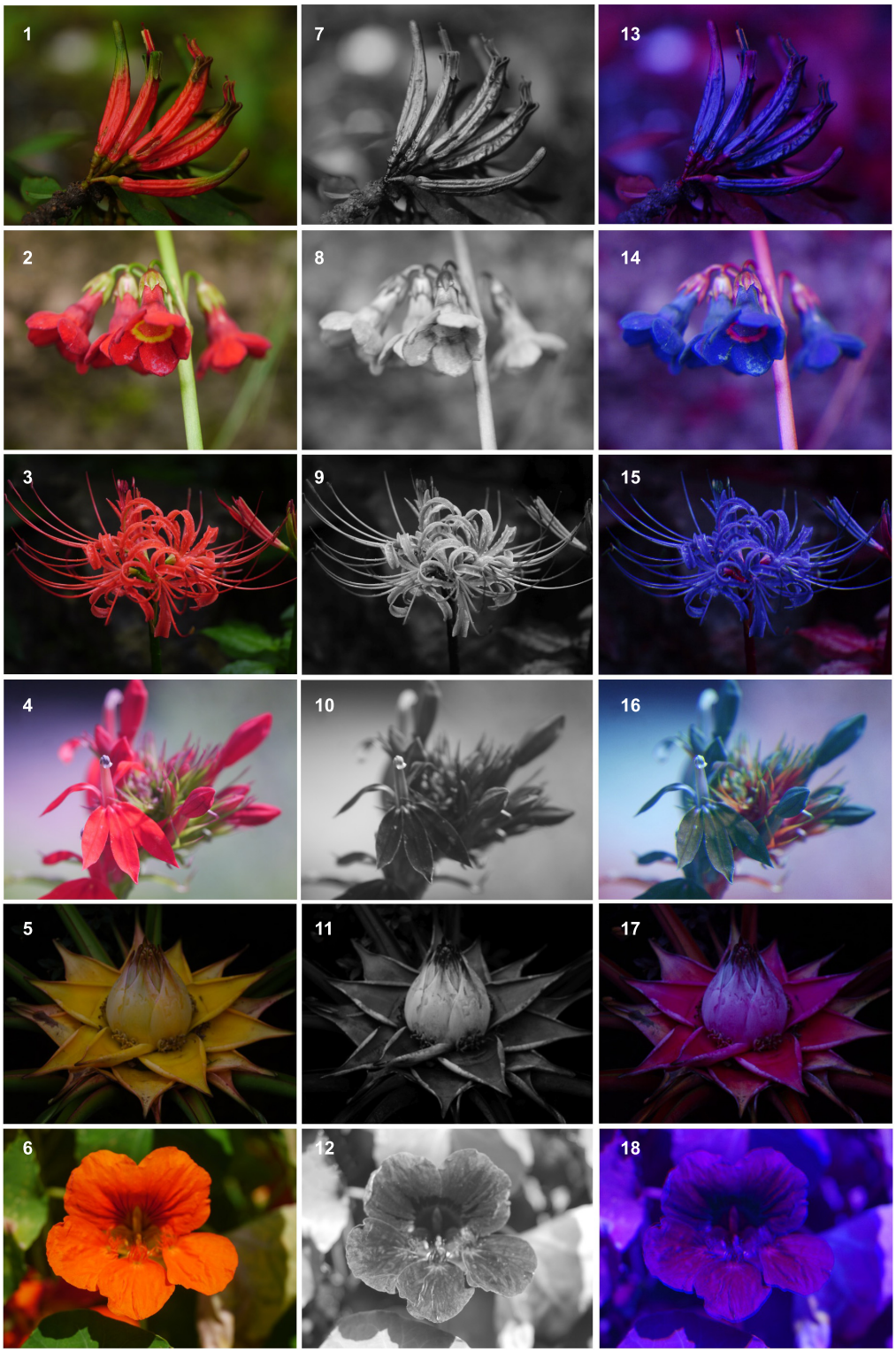


FIGURE 3 | Illustration of flower colors associated with the bird pollination syndrome highlighting the weak visual contrast to the background in bee vision. Color photos show flowers seen by humans (1–7), in ultraviolet (8–12) whereas false color photos in bee view consider the bee-visible range of wavelength and display ultraviolet as blue, blue as green and green as red, and red is discarded, the mixed color ultraviolet-blue is displayed as blue-green, blue-green is displayed as yellow, and purple is displayed as blue (13–18; Verhoeven et al., 2018). See **Figure 4** (2,8,14) and **Figure 4** (4,10,16) for comparison of background color contrast in a bumblebee-pollinated plant. Illustrated species are by row from top to bottom: *Taxillus caloreas* (Loranthaceae), *Primula anisodora* (Primulaceae), *Lycoris radiata* (Amaryllidaceae), *Lobelia cardinalis* (Campanulaceae), *Musella lasiocarpa* (Musaceae), *Tropaeolum majus* (Tropaeolaceae).

understanding of selection and adaptation of flower color. Moreover, contrast to surrounding foliage or substrate has been shown to play an important role for pollinator visual perception and detection of flowers (Bukovac et al., 2017), but has so far not been considered in analysis of pollinator-mediated selection.

In some cases, there may also be concurrent differences in multiple attributes of coloration within flowers, making it difficult to determine whether pollinator responses are associated with variation in one factor or another. For example, many yellow-flowered angiosperms have an ultraviolet-absorbing center, sometimes referred to as 'bull's eye' (Koski and Ashman, 2016). Alongside the UV-reflection, these bull's eyes often include shifts in pigment concentration resulting in a possible covariation between color hue with changes in brightness, saturation and contrast against the surrounding outer petals (Koski and Ashman, 2013, 2014). In such cases the direct target of selection may not be easily identified, and experimental trait manipulations may be needed to disentangle the character underlying pollinator attraction and behavior (Campbell et al., 2014; Koski and Ashman, 2014).

In summary, we conclude that flower color is more complex than previously acknowledged in most selection studies, and suggest the following:

- A comprehensive characterization of flower colors requires the combined knowledge of chemistry and physics. Chemistry is necessary to understand pigment concentration and composition. Physics is required to understand absorption, transmission, light reflection and backscattering, and structural colors based on surface properties rather than on pigment layers (van der Kooi et al., 2016).
- Future research should be directed towards redefining pollinator behavior as responses to signals and targets of higher conspicuousness through spectral purity (i.e., higher color saturation rather than hue) and higher contrast against the background of the plant vegetative parts and the surrounding vegetation (specifically green contrast and color contrast).
- Within-flower color transitions and contrast seem also promising targets of pollinator-mediated selection, that should be increasingly explored.
- Finally, future studies may also test if structural color properties of flowers are direct targets of selection by animal pollinators and evolved to aid plant-pollinator communication.

Pollinator Vision and Perception of Flower Color

The likelihood that a pollinator perceives a flower and eventually discriminates it from others will depend on complex interactions between various aspects of flower coloration, and the animals' sensory system and associated cognitive abilities (Chittka and Menzel, 1992; Chittka and Raine, 2006). Pollinator detection of a flower will then depend on the distance and direction to the flower, on a variety of floral and plant attractive characters, and on the surrounding sensory landscape such as vegetation context, ambient weather and light conditions (Dyer and Chittka, 2004a; Chittka and Raine, 2006; Dyer, 2006; Skorupski et al., 2006).

How an animal perceives the color of a flower depends on the spectral sensitivity of its photoreceptors, the number of photoreceptors, and the neural processing of the received spectral signal (Dyer et al., 2011; van der Kooi et al., 2021). For example, it is now well established that Hymenopteran vision is shifted towards shorter wavelengths compared to human vision with peak sensitivities occurring at 340, 430, and 535 nm enabling most bee species to perceive UV (Peitsch et al., 1992, **Supplementary Table 4**; **Figures 4**, **5**). The tetrachromatic vision in flies is based on two morphological receptor tandems (i.e., anatomically linked, consecutively laying pairs of photoreceptors), but they are lacking sensitivity to red light (Troje, 1993; An et al., 2018). Birds are generally tetrachromatic, but can be grouped into violet-sensitive species and UV-sensitive species, and both are sensitive to red light (Hart and Hunt, 2007). Butterflies' vision is even more complex and diverse than this with trichromatic, tetrachromatic or even higher dimensional color vision occurring depending on the species (Arikawa, 2017). The spectral sensitivities of the photoreceptor types have been identified in some species belonging to each of the most common flower-visiting functional group including bees, butterflies, hawkmoths, flies, birds, bats, and beetles (see **Supplementary Table 4** for an overview).

Thus, in contrast to other plant morphological characters, flower color is strictly speaking not simply a plants' character, but a neural interpretation of the chemical and physical properties of a flower as interpreted through an animal's visual system (i.e., a flower gets its colors only through the perceptual and cognitive process of its beholders; Garcia et al., 2020). For example, **Figures 3**, **4** illustrate flowers as seen by humans and by Hymenopterans with striking differences not only in hue but also in contrast among differently colored flower parts and contrast to green background foliage.

Models of animal vision can help us to understand how different kinds of pollinator functional groups might perceive flower color, and how similar or dissimilar two colors appear to a certain pollinator, and thus play a role for interpreting flower color discrimination and preferences. Models of animal vision have been formulated for a variety of pollinator taxa. For example, a pioneer achievement in this direction was the development of vision models to graphically represent the perceptual distances between loci in a color space, such as the frequently used color hexagon model for hymenopterans (Chittka, 1992; Chittka et al., 1994). The model visualizes the excitations from the three hymenopteran photoreceptors into a hexagon-shaped color space in the way that spectral reflectance data of flowers are transformed into units of discrimination in the trichromatic vision of a bee. Similar vision models have also been developed for flies (Troje, 1993) and butterflies (Koshitaka et al., 2008).

The vision models for hymenopteran, lepidopteran, and avian pollinators seem well aligned with results from behavioral studies (Vorobyev and Osorio, 1998; Vorobyev et al., 2001; Koshitaka et al., 2008; Kelber and Osorio, 2010), generally justifying their application in the study of pollinator-mediated



FIGURE 4 | Variation of flower color seen through the eyes of humans (1–6), in ultraviolet (7–12) and seen through the eyes of bees (12–18) using false color photography illustrating potential causes of neglected variation in flower color and bias in choice of study system. For a description of color representation see legend of **Figure 3**. Illustrated species are by row from top to bottom: *Anemone trullifolia* var. *holophylla* (Ranunculaceae), *Tibetia yunnanensis* (Fabaceae), *Roscoea schneideriana* (Zingiberaceae), *Pedicularis superba* (Orobanchaceae), *Anaphalis nepalensis* (Asteraceae), *Caltha palustris* (Ranunculaceae).

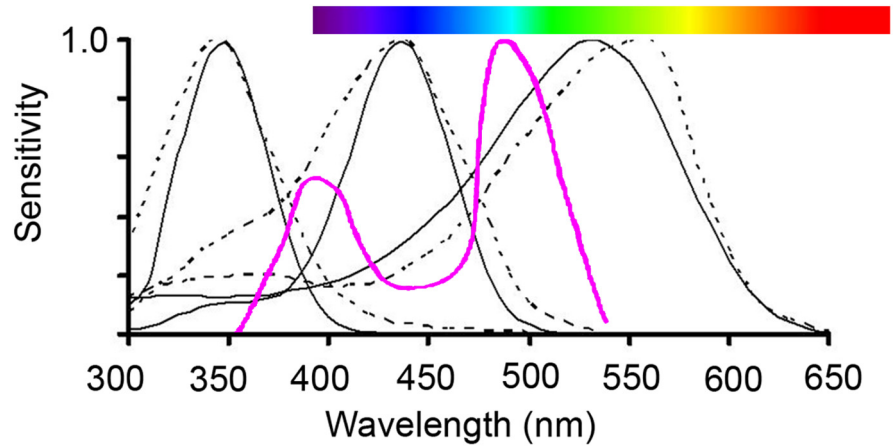


FIGURE 5 | Illustration of trichromatic vision of important hymenopteran pollinators, bumblebees (solid line) and honeybees (dotted line), which have photoreceptor sensitivities peaking in the UV range (about 350 nm), in the blue range (about 440 nm) and in the green range (540 nm). Human color vision is given as a reference above in the horizontal spectral visible bar. The purple line (inverted $\Delta\lambda/\lambda$ function for the honeybee; von Helversen, 1972; Chittka and Menzel, 1992) presents the two regions (400 nm and 500 nm), where spectral sensitivity curves overlap and therefore color discrimination can be expected to be highest. Graph reproduced from Shrestha et al. (2013).

selection. For example, four selection studies have used hexagon model transformation of spectral reflectance data or RGB values to estimate selection based on hexagon units of the color discrimination function. Moreover, Renoult et al. (2013) used this approach to show that selection on flower color brightness is a result of the hymenopteran visual system using a modeling approach (**Supplementary Table S3**).

However, models based on color spaces (e.g., color hexagon model) may be criticized as having low reliability, because they are based on a number of simplified and unrealistic assumptions and approximations (Osorio and Vorobyev, 2008; Shrestha et al., 2013). For example, although spectral sensitivity data are available for a diversity of bee species (Peitsch et al., 1992; **Supplementary Table 4**), most model calculations are done without using the specific spectral sensitivities of the investigated hymenopteran species, but by using the spectral sensitivity functions of the Western honeybee (*Apis mellifera*) or the buff-tailed bumblebee (*Bombus terrestris*) as an approximation. While some researchers argue that this generalized hexagon model of bee vision is likely applicable for a large number of hymenopteran pollinators (Briscoe and Chittka, 2001), because the spectral sensitivities of hymenopterans are (with few exceptions) similar (Peitsch et al., 1992; **Supplementary Table 4**), others argue it harbors severe limitations (Shrestha et al., 2013). Finally, it has been repeatedly demonstrated that the cognitive processes are not fixed but can be modulated in the course of foraging (Chittka et al., 2003; Dyer et al., 2012; further discussed below), and in response to spatially or temporally variable sensory landscapes (Koski, 2020). Therefore, color perception is perhaps more labile than any such vision model suggests.

Ideally, researchers would find that their results hold robust independent of the color vision model used (Telles and Rodríguez-Gironés, 2015; Gawryszewski, 2018), but so far such consistency has not always been found. For example, of those studies that have transformed flower color raw data into pollinator perception using the hexagon model (Campbell et al., 2012; Renoult et al., 2013; Joffard et al., 2020; **Supplementary Table 3**), some found no difference between selection estimated using vision model transformed data and raw data (Joffard et al., 2020), while others did find differences (Brunet et al., 2021). As far as we know, it is not known what explains these deviations (e.g., Brunet et al., 2021) and further research is needed to identify the factors governing such inconsistencies. Interestingly, Sletvold et al. (2016) and Brunet et al. (2021) found significant selection by pollinators on attributes of flower color without the use of any vision model but by demonstrating through supplemental hand-pollination and observation of pollinator behavior, respectively, that selection is a result of differential pollinator interactions.

Pollinators can only respond to variation among flower colors when they perceive the differences, and pollinator discrimination of certain colors may be constrained by the pollinator's capacity for distinctive color perception. It has, for example, been demonstrated that bumblebees (*Bombus terrestris*) can learn to distinguish colors separated by 0.045 hexagon units (Dyer and Chittka, 2004b) and honeybees distinguish monochromatic stimuli separated by 4.5 nm (von Helversen, 1972). This suggests that Hymenopterans (the dominant pollinators in most selection studies) should be able to perceive the variation present in many natural plant populations of a single species (Campbell et al., 2012; Renoult et al., 2013).

However, these thresholds of minimal detectable differences were obtained under laboratory conditions and it is questionable whether such fine scale color discrimination applies to the foraging behavior of bees in natural floral communities with varying backgrounds and illuminations (Chittka and Thomson, 2001; Dyer and Chittka, 2004b; Dyer, 2006; Skorupski et al., 2006). For example, Dyer and Chittka (2004a) showed

that illumination can affect bees color perception and the discrimination of fine color gradients: the number of correct choices of near similar colors increased with experience and with the color distance between the colors and was dependent on the ambient illumination as predicted by color vision model calculations.

Generally, laboratory behavioral experiments suggest, that fine color discrimination requires an association of the colored target with a reward. For example, depending on the experimental associations of color stimuli and rewards, Dyer and Chittka (2004b) showed that the ability of honeybees to discriminate similar colors can vary by about a factor of three. For free foraging bumblebees to specialize over flower colors, Chittka et al. (1997) suggest that, these colors must be separated by at least 0.1 hexagon units.

Yet, a strong consistent color-reward relationship as offered in laboratory settings is unlikely to occur in natural populations (Parachnowitsch et al., 2019; see discussion below). This may explain partly the deviation between the outcomes of experiments to identify the maximal possible visual capacities of bees, and those quantifying selection pressure on flower color in natural plant populations based on bee preferential flower visitation.

To help resolve the issues identified above in the context of quantification of pollinator-mediated selection, we make the following suggestions:

- Comparative spectral sensitivity data for model and non-model species of pollinators are needed. This would enable us to better understand the diversity of insect color perception, both among and within populations, before continuing to apply vision models. In worst case scenarios, such models only imprecisely fit the focal pollinator species.
- Measuring selection is combined with an approach to link variation in plant fitness to variation in pollinator preference and behavior (e.g., experimentally manipulating the pollination environment, structural equation modeling). It should then be possible to evaluate relationships between color phenotype and fitness (i.e., the target, mode and strength of selection), as mediated by pollinator interactions, without the use of vision models.

Pollinator Attraction and Response to Flower Color

A pollinator may detect and respond to flower colors at a range of distances, with different color attributes coming into play as distances range from far to near, and ultimately when the pollinator reaches and moves onto a flower. For example, in bees, achromatic flower color is used for far distance detection of flowers, while chromatic flower color becomes important only when they are already in close proximity to them or flower targets are large-sized (Hempel de Ibarra et al., 2015). Only at relatively close distances (<10 cm), fine discrimination of chromatic colors between flowers is possible and enables expression of pollinator preferential choices to visit a specific flower phenotype (Giurfa et al., 1996; Hempel de Ibarra et al., 2015). Upon attraction, within-flower color patterns (e.g., floral guides) may become important through manipulating pollinator behavior close to or inside the flower (sensu Pohl et al., 2008; Pyke, 2016; Leonard and Papaj, 2011).

Different aspects of flower color may be subject to correlational selection through the effects on the number of fruits produced per plant and the seeds produced per flower. Flower achromatic and chromatic colors may determine the number of visits to a flower and thereby fruit production, while flower color patterning can influence pollinator orientation within the flower and flower handling time (Leonard and Papaj, 2011) as well as their feeding behavior such as the likelihood of proboscis extension (Hansen et al., 2012; Bischoff et al., 2015). Such patterns may act as 'guides' towards a potential resource (e.g., food, mating opportunity) and the reproductive organs, and thereby can influence the efficiency of pollen removal and deposition.

Pollinator responses to flower color signals may be innate, that is the spontaneous attraction of naïve animals as a consequence of their specific genetically determined visual systems (reviewed in Lunau and Maier, 1995). Such preferential innate attraction to certain color attributes is illustrated, by example, for preference of bees for blue or purple and some hoverflies for yellow signals (Lunau and Maier, 1995), while nocturnal moths' preference for white flowers (Goyret et al., 2008) and hummingbirds' preference for red flowers (Lunau et al., 2011) is not necessarily by innate response. Innate preferences are not necessarily associated with a specific hue. For example, a series of choice experiments with naïve bees and artificial flowers of varying colors have demonstrated that bees discriminate colors according to differences in hue, but spontaneously prefer colors of higher saturation independently of color hue (Lunau, 1990; Lunau and Maier, 1995; Lunau et al., 1996).

Several studies have demonstrated that almost any flower-visiting animal can be trained to respond to almost any flower color that is associated with a reward suggesting that most flower color preferences are extremely labile and can readily be changed through experience, and associative learning of a signal with a reward (Lunau and Maier, 1995; Goyret et al., 2008). For example, nectar-feeding hummingbirds learn to associate a particular flower color with relatively abundant nectar, and subsequently prefer to visit flowers with this color (Collias and Collias, 1968; Goldsmith and Goldsmith, 1979). A striking example is also provided by *Manduca* moths which innately prefer blue flowers but, just like other nocturnal pollinators, they are mainly seen foraging on white flowers in the wild (Goyret et al., 2008).

Labile color preferences have been shown for many animal pollinators including Hymenoptera, Lepidoptera, Diptera and Aves, allowing them to easily generalize in their foraging over a large array of flower colors (Lunau and Maier, 1995). This suggests that color preferences are dynamically formed and hence may exhibit variation that is temporal (Vaknin et al., 1996; Souza et al., 2018), or depends on flower patches of variable local co-flowering community (i.e., small scale spatial variation in preference; Wassink and Caruso, 2013).

In contrast to innate responses, pollinator color preference should depend on the flower color- reward relationship within the focal population and within the surrounding co-flowering community (Waser and Price, 1981; Campbell et al., 1997;

Jersáková et al., 2016). If indeed flower color acts as a reliable signaling cue for pollinators in search for a reward, it provides a realistic mechanism explaining pollinator-mediated selection on flower color. However, none of the selection studies estimated the color-reward relationship and its temporal variation within the study population and the co-flowering community. Associations between color and nectar traits might be relevant across plant lineages explaining pollinator shifts, but may be less expected to occur within populations (Waser and Price, 1981; Parachnowitsch et al., 2019; Brunet et al., 2021). This is because pollinators are constantly depleting floral resources, flowers are refilling resources and there are temporal dynamics of flower abundance and community composition, and so it is unlikely that a consistent correlation between flower color and flower reward is prevalent in many plant populations and communities (Waser and Price, 1981). In fact, one of the arguments to explain the commonly found higher phenotypic variation in flowering traits among populations of non-rewarding species compared to populations of rewarding species is the lack of such a signal-reward association, which prevents pollinator learning of signals and consequently pollinator-mediated directional or stabilizing selection (Salzmann et al., 2007; Dormont et al., 2019). However, this hypothesis is contrasted by one of the few studies that detected significant pollinator-mediated selection on two flower color traits, which was conducted in an early-spring flowering non-rewarding orchid. This species begins to flower before any rewarding species comes to bloom and is pollinated by naïve bumblebee queens that have not yet acquired the ability to use a learned floral signal, yet there was significant selection by these bees for increased brightness and color contrast and the factors that maintain the large intra-population variation remain unknown (Sletvold et al., 2016).

The ability of animals to detect the color of plants and flowers depends on the visual context as well as the behavior of the animal. For example, Dyer and Chittka (2004a) showed that the ability to perceive and discriminate fine color differences (measured as the number of correct choices of near similar colors) decreased at lower illumination. However, this reduction in performance was less when bees were experienced and when the color distance between the stimuli was larger. Furthermore, the specific pollinators' behavior can influence the detection when, for example, bees forfeit their abilities to discriminate colors in favor of making speedy decisions (Chittka et al., 2003; Skorupski et al., 2006).

We conclude that, the context-dependence of pollinator foraging challenges the linking of results of pollinator preferential behavior for flower colors as obtained under laboratory conditions to the interactions between natural plant and pollinator populations (Ng et al., 2020), and therefore suggest that:

- Future studies should test how results from laboratory studies are related to the foraging behavior of wild pollinators, and explore the factors that may explain possible deviations.
- Studies in the wild should further explore the context-dependence of pollinator preferential foraging using flower color in relation to experience and acquisition of color-reward relationships within flowering populations and communities.

Pollinator-Mediated Selection on Flowering Traits

The direction, shape and strength of selection depends on, first, the functional relationships between traits and fitness, and second, the magnitude of variation in associated fitness. In the case of flower color, these relationships will be determined, as discussed above, by pollinator color perception, as well as their cognitive and behavioral responses. Therefore, the extent of within-population variation in flower color traits, and the way it affects variation in the interaction with pollinators, should determine the realized pollinator-mediated selection observed in natural populations. Consequently, the shape and strength of pollinator-mediated selection depends on the variation in the phenotypic character and its effect on pollen receipt and pollen export (Trunschke et al., 2020).

Pollinator-mediated selection will depend on the abilities of pollinators to discriminate between different colors and these may be influenced by a number of factors. For example, even if some bee pollinators do perceive differences in wavelength down to 0.045 hexagon units (see discussion in preceeding section; Dyer and Chittka, 2004b), this threshold of discrimination ability in bees is not equal over the spectral range of flower colors (Chittka and Waser, 1997; **Figure 5**). In general, pollinators should show highest discrimination capacities when two photoreceptors are maximum excited simultaneously, which explains the poor capacities of most bees to contrast red against their surroundings (Chittka and Waser, 1997; **Figures 4**, **5**).

The spectral reflectance curves of flowers are striking because they possess sharp transitions in reflectance and because the position of these so-called 'spectral reflectance' marker points cluster in small ranges of wavelengths (Shrestha et al., 2013; Dorin et al., 2020; **Figure 5**). For example, it has been argued that hymenopteran spectral sensitivities of photoreceptor types are located in the wavelength spectrum such that they generate the largest possible range of different excitation values for inter-specific flower signals, and thus improve color discrimination among species within flowering communities (Chittka and Menzel, 1992). However, whether optimally foraging bees use fine-tuned color discrimination to distinguish between flower colors with similar marker points is not clear.

We may therefore expect that differential pollinator visitation is most expressed when intra-population color variation is either extremely large (e.g., Campbell et al., 1997; Hirota et al., 2013; Sletvold et al., 2016), or variation occurs in the range of maximum discrimination capacities. For example, selection in bee-pollinated plant species can be expected to be weak on chromatic signals in the yellowish to reddish range where subtle color variation cannot be well perceived (Chittka and Waser, 1997; **Figure 5**). This is in line with the lack of selection by bumblebee pollinators in several orange and yellow-flowered populations of *Gentiana lutea* (Sobral et al., 2015; **Table 2**). Further, if

variation in flower color occurs in a range of the phenotypic distribution where it has little effect on the variation in fitness, this can result in weak selection limiting its detection (Trunschke et al., 2020).

Selection for flower colors could be stronger for plants with specialized pollinator interactions compared with plants utilized by pollinators belonging to several different taxonomic groups. It might be possible, for example, to predict the direction and shape of selection of flower color for specialized pollinator interactions, where there is a single main pollinator species with a particular operating visual system (Renoult et al., 2013). On the other hand, flower color selection may be less precise for plant species that are served by multiple pollinating animal species, which differ in their operating visual systems (Campbell et al., 1997), or their responses to perceived signals (Brunet et al., 2021).

Flower color may correlate with other traits influencing the number of pollinator visits and pollination efficiency (Gómez, 2000; Armbruster, 2002). Such traits may include overall plant stature and flower morphology (Gómez, 2000; Frey et al., 2011; Rodriguez-Castañeda et al., 2020), or, due to possible linkage in biosynthetic pathways, floral scent (Majetic et al., 2007; Zvi et al., 2008; Dormont et al., 2019 and references therein). Depending on the genetic variance-covariance matrix for all traits involved in pollinator interaction in a population, selection on flower color can therefore also act indirectly through pleiotropic links or in a correlative fashion favoring trait integration (Armbruster, 2002; Strauss and Whittall, 2006; Rausher, 2008).

Amongst a number of plant traits that may attract pollinators, one of the strongest predictors of pollinator visitation and pollination success is the size of floral displays (most commonly as the number of open flowers). This is because the number of flowers an individual plant produces strongly correlates with the quantity of flower rewards (Parachnowitsch et al., 2019), and therefore provides a reliable cue for pollinators to assess the amount of resource. Indeed, one study detected correlational selection between flower color and floral display by bumblebees favoring larger and darker flowers in *Medicago sativa* (Brunet et al., 2021). In contrast, most selection studies discussed here did not observe significant trait correlations between color and morphology, and have not explored this possibility further (**Table 1**). Few studies have compared selection differentials, which estimate both direct and indirect selection on a quantitative character, and selection gradients which reveal directional selection (Lande and Arnold, 1983; Arnold and Wade, 1984). These studies found that selection differentials are not largely different from selection gradients (**Supplementary Table 5**), suggesting that flower color is a direct target of selection in these studies.

In plants relying on animal pollination, the extent to which seed production is limited by pollination (i.e., the degree of pollen limitation) further influences the strength of pollinator-mediated selection. This is because pollen limitation influences the variation in fitness among individuals (i.e., the opportunity for selection; Sletvold and Ågren, 2014; Bartkowska and Johnston, 2015; Trunschke et al., 2017) and further, because pollen limitation can influence the trait-fitness relationship (discussed above). By definition pollen limitation quantifies the intensity of interactions with pollinators in the way that under high pollinator abundance, variation in pollination success among flowering individuals can be expected to be low, whereas under low pollinator abundancy the opposite occurs. Moreover, under high pollinator abundancy, competition among pollinators for floral resources may increase, which could lead pollinators to change their individual foraging strategy towards visiting an otherwise less preferred color phenotype (Waser and Price, 1981). Consistent with this prediction, the strongest selection on a flower color signal was found in the study population characterized by a high degree of pollen limitation (PL = 0.89 in the year of study; Sletvold et al., 2016). In other studies pollen limitation is often not reported, but fruit or seed set appears to be rather intermediate or high, which may explain weak detected selection.

In summary, we suggest that future studies should:

- Characterize functional relationships between flower color attributes and pollinator visitation and efficiency.
- Quantify how this translates into pollinator-mediated selection.
- Investigate the influence of the pollination environment including the rewarding co-flowering community and the magnitude of pollen limitation.

Such studies, with experimentally increased phenotypic variation, should elucidate the adaptive value of flower colors within contemporary populations, whereas manipulation of the environmental context should provide insights into the factors underlying variation in pollinators selecting for flower color.

CONCLUSION

Quantifying selection acting on continuous flower color variation including the assessment of pollinator-mediated selection is necessary for understanding the importance of pollinator interactions for macro- and micro-evolutionary patterns and processes in the evolution and divergence of flower color in angiosperms. Clearly, more studies are needed that identify the direct targets, and characterize the direction, form and strength of selection on flower color signals within and among populations, and among species within flowering plant communities.

In this review, we have highlighted that while macro-evolutionary patterns for pollinator-driven evolution of flower color are well established and accepted, little is known about the underlying micro-evolutionary processes of pollinator-mediated selection within natural populations. Few studies have demonstrated that pollinators can be agents of selection on flower color, and detecting such selection appears to be challenging. This difficulty arises for multiple reasons related to the methodology of visualization and recognition of the color properties under selection, the likely flexibility of the trait-fitness relationship as response of pollinator perception, cognition and behavior, and

the dependence of pollinator mediated selection on the pollination environment.

The evolution of floral signals in response to pollinator interactions is a complex field, that requires knowledge in botany, zoology, evolutionary biology, behavioral ecology as well as sensory physiology and neurobiology. Similar to floral scent blends, flower color should be recognized as a receiver-dependent and complex trait, the study of which requires an interdisciplinary approach. To date, this is seldomly played out in practice, and we strongly encourage scientists from animal and plant research to cross borders and work collaboratively.

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All authors participated in the initial discussion for the manuscript. JT drafted a first version and led the writing, and all authors revised subsequent versions.

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SUPPLEMENTARY MATERIAL

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REFERENCES

An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly *Eristalis tenax* prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 21:221. doi: 10.1242/jeb.184788

Arikawa, K. (2017). The eyes and vision of butterflies. *J. Physiol.* 595, 5457–5464. doi: 10.1113/JP273917

Armbruster, W. S. (2002). Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *J. Evol. Biol.* 15, 468–486. doi: 10.1046/j.1420-9101.2002.00399.x

Arnold, S. E. J., and Chittka, L. (2012). Illumination preference, illumination constancy and colour discrimination by bumblebees in an environment with patchy light. *J. Exp. Biol.* 215, 2173–2180. doi: 10.1242/jeb.065565

Arnold, S. J., and Wade, M. J. (1984). On the measurement of natural and sexual selection: theory. *Evolution* 38, 709–719. doi: 10.1111/j.1558-5646.1984.tb00344.x

Bartkowska, M. P., and Johnston, M. O. (2015). Pollen limitation and its influence on natural selection through seed set. *J. Evol. Biol.* 28, 2097–2105. doi: 10.1111/jeb.12741

Bischoff, M., Raguso, R. A., Jürgens, A., and Campbell, D. R. (2015). Context-dependent reproductive isolation mediated by floral scent and color. *Evolution* 69, 1–13. doi: 10.1111/evo.12558

Briscoe, A. D., and Chittka, L. (2001). The evolution of color vision in insects. *Ann. Rev. Entomol.* 46, 471–510. doi: 10.1146/annurev.ento.46.1.471

Brunet, J., Flick, A. J., and Bauer, A. A. (2021). Phenotypic selection on flower color and floral display size by three bee species. *Front. Plant Sci.* 11:587528. doi: 10.3389/fpls.2020.587528

Bukovac, Z., Shresta, M., Garcia, J. E., Burd, M., Dorin, A., and Dyer, A. G. (2017). Why background color matters to bees and flowers. *J. Comp. Physiol.* 203, 369–380. doi: 10.1007/s00359-017-1175-7

Campbell, D. R., Bischoff, M., Lord, J. M., and Robertson, A. W. (2012). Where have all the blue flowers gone: pollinator responses and selection on flower colour in New Zealand *Wahlenbergia albomarginata*. *J. Evol. Biol.* 25, 352–364. doi: 10.1111/j.1420-9101.2011.02430.x

Campbell, D. R., Forster, M., and Bischoff, M. (2014). Selection of trait combinations through bee and fly visitation to flowers of *Polemonium foliosissimum*. *J. Evol. Biol.* 27, 325–336. doi: 10.1111/jeb.12295

Campbell, D. R., Waser, N. M., and Meléndez-Ackerman, E. J. (1997). Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *Am. Nat.* 149, 295–315. doi: 10.1086/285991

Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2018). A meta-analysis of the agents of selection on floral traits. *Evolution* 73, 4–14. doi: 10.1111/evo.13639

Caruso, C. M., Scott, S. L., Wray, J. C., and Walsh, C. A. (2010). Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *Int. J. Plant Sci.* 171, 1020–1028. doi: 10.1086/656511

Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331

Chittka, L. (1997). Bee color vision is optimal for coding flower color, but flower colors are not optimal for being coded – Why? *Isr. J. Plant Sci.* 45, 115–127. doi: 10.1080/07929978.1997.10676678

Chittka, L., Dyer, A. G., Bock, F., and Dornhaus, A. (2003). Psychophysics: bees trade off foraging speed for accuracy. *Nature* 424:388. doi: 10.1038/424388a

Chittka, L., Gumbert, A., and Kunze, J. (1997). Foraging dynamics in bumble bees: correlates of movements within and between plant species. *Behav. Ecol.* 8, 239–249. doi: 10.1093/beheco/8.3.239

Chittka, L., and Menzel, R. (1992). The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J. Comp. Physiol. A* 171, 171–181. doi: 10.1007/BF00188925

Chittka, L., and Raine, N. E. (2006). Recognition of flowers by pollinators. *Curr. Opin. Plant Biol.* 9, 428–435. doi: 10.1016/j.pbi.2006.05.002

Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of Hymenopteran. *Vision Res.* 34, 1489–1508. doi: 10.1016/0042-6989(94)90151-1

Chittka, L., and Thomson, J. D. (2001). *Cognitive Ecology of Pollination*. New York, NY: Cambridge University Press. doi: 10.1017/CBO9780511542268

Chittka, L., and Waser, N. M. (1997). Why red flowers are not invisible to bees. *Isr. J. Plant Sci.* 45, 169–183. doi: 10.1080/07929978.1997.10676682

Collias, N. E., and Collias, E. C. (1968). Anna's Hummingbirds trained to select different colors in feeding. *Condor* 70, 273–274. doi: 10.2307/1366705

Corbet, S. A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A., et al. (1993). Temperature and the pollinating activity of social bees. *Ecol. Entomol.* 18, 17–30. doi: 10.1111/j.1365-2311.1993.tb01075.x

Dorin, A., Shresta, M., Herrmann, M., Burd, M., and Dyer, A. G. (2020). Automated calculation of spectral reflectance marker points to enable analysis of plant colour-signalling to pollinators. *MethodsX* 7:100827. doi: 10.1016/j.mex.2020.100827

Dormont, L., Joffard, N., and Schatz, B. (2019). Intraspecific variation in floral color and odor in orchids. *Int. J. Plant Sci.* 180, 1036–1058. doi: 10.1086/705589

Dyer, A. G. (2006). Discrimination of flower colours in natural settings by the bumblebee species *Bombus terrestris* (Hymenoptera: Apidae). *Entomol. Gen.* 28, 257–268. doi: 10.1127/entom.gen/28/2006/257

Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V., and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. Lond. B* 279, 3606–3615. doi: 10.1098/rspb.2012.0827

Dyer, A. G., and Chittka, L. (2004a). Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comp. Physiol. A* 190, 105–114. doi: 10.1007/s00359-003-0475-2

Dyer, A. G., and Chittka, L. (2004b). Fine colour discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91, 224–227. doi: 10.1007/s00114-004-0508-x

Dyer, A. G., Paulk, A. C., and Reser, D. H. (2011). Colour processing in complex environments: insights from the visual system of bees. *Proc. R. Soc. Lond. B* 278, 952–959. doi: 10.1098/rspb.2010.2412

Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.3732/ajb.0800222

Frey, F. M. (2004). Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58, 2426–2437. doi: 10.1111/j.0014-3820.2004.tb00872.x

Frey, F. M., Dunton, J., and Garland, K. (2011). Floral color variation and associations with fitness-related traits in *Malva moschata* (Malvacese). *Plant Species Biol.* 26, 235–243. doi: 10.1111/j.1442-1984.2011.00325.x

Garcia, J. E., Greentree, A. D., Shrestha, M., Dorin, A., and Dyer, A. G. (2014). Flower colours through the lens: quantitative measurement with visible and ultraviolet Digital Photography. *PLoS One* 9:e96646. doi: 10.1371/journal.pone.0096646

Garcia, J. E., Phillips, R. D., Peter, C. I., and Dyer, A. G. (2020). Changing how biologists view flowers – color as perception not a trait. *Front. Plant Sci.* 11:601700. doi: 10.3389/fpls.2020.601700

Garcia, J. E., Shrestha, M., Howard, S. R., Petersen, P., and Dyer, A. G. (2019). Signal or cue: the role of structural colors in flower pollination. *Curr. Zool.* 65, 467–481. doi: 10.1093/cz/zoy096

Gawryszewski, F. M. (2018). Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. *Ecol. Evol.* 8, 8159–8170. doi: 10.1002/ece3.4288

Gervasi, D. D. L., and Schiestl, F. P. (2017). Real-time divergent evolution in plants driven by pollinators. *Nat. Commun.* 8:14691. doi: 10.1038/ncomms14691

Giurfa, M., Vorobyev, M., Kevan, P., and Menzel, R. (1996). Detection of coloured stimuli by honey bees: minimum visual angles and receptor specific contrasts. *J. Comp. Physiol. A* 178, 699–709. doi: 10.1007/BF00227381

Goldsmith, T. H., and Goldsmith, K. M. (1979). Discrimination of colors by the black-chinned hummingbird, *Archilochus alexandri*. *J. Comp. Physiol. A* 130, 209–220. doi: 10.1007/BF00614607

Gómez, J. M. (2000). Phenotypic selection and response to selection in *Lobularia maritima* importance of direct and correlational components of natural selection. *J. Evol. Biol.* 13, 689–699.

Goyret, J., Pfaff, M., Raguso, R., and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learned color preferences in a hawkmoth. *Naturwissenschaften* 95, 569–576. doi: 10.1007/s00114-008-0350-7

Gross, K., Sun, M., and Schiestl, F. P. (2016). Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. *PLoS One* 11:e0147975. doi: 10.1371/journal.pone.0147975

Hansen, D. M., Van der Niet, T., and Johnson, S. D. (2012). Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proc. R. Soc. Lond. B* 279, 634–639. doi: 10.1098/rspb.2011.1349

Hart, N. S., and Hunt, D. M. (2007). Avian visual pigments: characteristics, spectral tuning, and evolution. *Am. Nat.* 169, 7–26. doi: 10.1086/510141

Hempel de Ibarra, N., Langridge, K. V., and Vorobyev, M. (2015). More than colour attraction: behavioural functions of flower patterns. *Curr. Opin. Insect Sci.* 12, 64–70. doi: 10.1016/j.cois.2015.09.005

Hirota, S. K., Nitta, K., Suyama, Y., Kawakubo, N., Yasumoto, A. A., and Yahara, T. (2013). Pollinator-mediated selection on flower color, flower scent and flower morphology of *Hemerocallis*: evidence from genotyping individual pollen grains on the stigma. *PLoS One* 8:e85601. doi: 10.1371/journal.pone.0085601

Horth, L., Campbell, L., and Bray, R. (2014). Wild bees preferentially visit *Rudbeckia flower* heads with exaggerated ultraviolet absorbing floral guides. *Biol. Open* 3, 221–230. doi: 10.1242/bio.20146445

Hsu, H.-C., Hsu, K.-L., Chan, C.-Y., Wang, C.-N., and Kuo, Y.-F. (2018). Quantifying colour and spot characteristics for the ventral petals in *Sinningia speciosa*. *Biosyst. Eng.* 167, 40–50. doi: 10.1016/j.biosystemseng.2017.12.010

Iriel, A., and Lagorio, M. G. (2010). Is the flower fluorescence relevant in biocommunication? *Naturwissenschaften* 97, 915–924. doi: 10.1007/s00114-010-0709-4

Irwin, R. E., and Strauss, S. Y. (2005). Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am. Nat.* 165, 225–237. doi: 10.1086/426714

Jersáková, J., Spaethe, J., Streinzer, M., Neumayer, J., Paulus, H., Dötterl, S., et al. (2016). Does *Traunsteinera globose* (the globe orchid) dupe its pollinators through generalized food deception or mimicry? *Bot. J. Linn. Soc.* 180, 269–294. doi: 10.1111/boj.12364

Joffard, N., Le Roncé, I., Langlois, A., Renoult, J., Buatois, B., Dormont, L., et al. (2020). Floral trait differentiation in *Anacamptis coriophora*: phenotypic selection on scents, but not on colour. *J. Evol. Biol.* 33, 1028–1038. doi: 10.1111/jeb.13657

Johnsen, S. (2016). How to measure color using spectrometers and calibrated photographs. *J. Exp. Biol.* 219, 772–778. doi: 10.1242/jeb.124008

Kelber, A., and Osorio, D. (2010). From spectral information to animal colour vision: experiments and concepts. *Proc. R. Soc. Lond. B* 277, 1617–1625. doi: 10.1098/rspb.2009.2118

Kjernsmo, K., Hall, J. R., Doyle, C., Khuzayim, N., Cuthill, I. C., Scott-Samuel, N. E., et al. (2018). Iridescence impairs object recognition in bumblebees. *Sci. Rep.* 8:8095. doi: 10.1038/s41598-018-26571-6

Koshitaka, H., Kinoshita, M., Vorobyev, M., and Arikawa, K. (2008). Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proc. R. Lond. Soc. B* 275, 947–954. doi: 10.1098/rspb.2007.1614

Koski, M. H. (2020). The role of sensory drive in floral evolution. *New Phytol.* 227, 1012–1024. doi: 10.1111/nph.16510

Koski, M. H., and Ashman, T.-L. (2013). Quantitative variation, heritability, and trait correlations for ultraviolet floral traits in *Argentina anserina* (Rosaceae): implications for floral evolution. *Int. J. Plant Sci.* 174, 1109–1120. doi: 10.1086/671803

Koski, M. H., and Ashman, T.-L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct. Ecol.* 28, 868–877. doi: 10.1111/1365-2435.12242

Koski, M. H., and Ashman, T.-L. (2016). Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors. *New Phytol.* 211, 708–718. doi: 10.1111/nph.13921

Kraaij, M., and van der Kooi, C. J. (2019). Surprising absence of association between flower surface microstructure and pollination system. *Plant Biol.* 22, 177–183. doi: 10.1111/plb.13071

Lande, R., and Arnold, S. (1983). The measurement of selection on correlated characters. *Evolution* 37, 1210–1226. doi: 10.1111/j.1558-5646.1983.tb00236.x

Lavi, R., and Sapir, Y. (2015). Are pollinators the agents of selection for extreme large size and dark color in *Oncocyclus* irises? *New Phytol.* 205, 369–377. doi: 10.1111/nph.12982

Lawson, D. A., and Rands, S. A. (2019). The effects of rainfall on plant-pollinator interactions. *Arthropod Plant Interact.* 13, 561–569. doi: 10.1007/s11829-019-09686-z

Leonard, A. S., and Papaj, D. R. (2011). 'X' marks the spot: the possible benefits of nectar guides to bees and plants. *Funct. Ecol.* 25, 1293–1301. doi: 10.1111/j.1365-2435.2011.01885.x

Lunau, K. (2000). The ecology and evolution of visual pollen signals. *Pl. Syst. Evol.* 222, 89–111. doi: 10.1007/BF00984097

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: flower dummy experiments with bumblebees. *J. Comp. Physiol. A* 166, 827–834. doi: 10.1007/BF00187329

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. *J. Comp. Physiol. A* 177, 1–19. doi: 10.1007/BF00243394

Lunau, K., Papiorek, S., Eltz, T., and Sazima, M. (2011). Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *J. Exp. Biol.* 214, 1607–1612. doi: 10.1242/jeb.052688

Lunau, K., Ren, Z.-X., Fan, X.-Q., Trunschke, J., Pyke, G. H., and Wang, H. (2020). Nectar mimicry: a new phenomenon. *Sci. Rep.* 10:7039. doi: 10.1038/s41598-020-63997-3

Lunau, K., Wacht, S., and Chittka, L. (1996). Colour choices of naïve bumble bees and their implications for colour perception. *J. Comp. Physiol. A* 178, 477–489. doi: 10.1007/BF00190178

Majetic, C. J., Raguso, R. A., Tonsor, S. J., and Ashman, T.-L. (2007). Flower color-scent associations in polymorphic *Hesperis matronalis* (Brassicaceae). *Phytochemistry* 68, 865–874. doi: 10.1016/j.phytochem.2006.12.009

Medel, R., Botto-Mahan, C., and Kalin-Arroyo, M. (2003). Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* 84, 1721–1732. doi: 10.1890/01-0688

Meléndez-Ackerman, E. J., Campbell, D. R., and Waser, N. M. (1997). Hummingbird behavior and mechanisms of selection on flower colour in *Ipomopsis*. *Ecology* 78, 2532–2541. doi: 10.1890/0012-9658(1997)078[2532:HBAMOS]2.0.CO;2

Menzel, R., Gumbert, A., Kunze, J., Shmida, A., and Vorobyev, M. (1997). Pollinator's strategies in finding flowers. *Isr. J. Plant Sci.* 45, 141–156. doi: 10.1080/07929978.1997.10676680

Menzel, R., and Shmida, A. (1993). The ecology of flower colours and the natural colour vision of insect pollinators: the Israeli flora a case study. *Biol. Rev.* 68, 81–120. doi: 10.1111/j.1469-185X.1993.tb00732.x

Mizuno, T., Uehara, A., Mizuta, D., Yabuya, T., and Iwashina, T. (2015). Contribution of anthocyanin–flavone copigmentation to grayed violet flower color of Dutch iris cultivar 'Tiger's Eye' under the presence of carotenoids. *Sci. Hortic.* 186, 201–206. doi: 10.1016/j.scienta.2015.01.037

Mori, S., Fukui, H., Oishi, M., Sakuma, M., Kawakami, M., Tsukioka, J., et al. (2018). Biocommunication between plants and pollinating insects through fluorescence of pollen and anthers. *J. Chem. Ecol.* 44, 591–600. doi: 10.1007/s10886-018-0958-9

Ng, L., Garcia, J. E., and Dyer, A. G. (2020). Why colour is complex: evidence that bees perceive neither brightness nor green contrast in colour signal processing. *Facets* 3, 800–817. doi: 10.1139/facets-2017-0116

Ohashi, K., Makino, T. T., and Arikawa, K. (2015). Floral color change in the eyes of pollinators: testing possible constraints and correlated evolution. *Funct. Ecol.* 29, 1144-1155. doi: 10.1111/1365-2435.12420

Osorio, D., and Vorobyev, M. (2008). A review of the evolution of animal colour vision and visual communication signals. *Vision Res.* 48, 2042–2051. doi: 10.1016/j.visres.2008.06.018

Papiorek, S., Junker, R. R., and Lunau, K. (2014). Gloss, colour and grip: multifunctional epidermal cell shapes in bee- and bird-pollinated flowers. *PLoS One* 9:e112013. doi: 10.1371/journal.pone.0112013

Papiorek, S., Rohde, K., and Lunau, K. (2013). Bees' subtle colour preferences: how bees respond to small changes in pigment concentration. *Naturwissenschaften* 100, 633–643. doi: 10.1007/s00114-013-1060-3

Parachnowitch, A. L., and Kessler, A. (2010). Pollinators exert natural selection on flower size and floral display in Penstemon digitalis. *New Phytol.* 188, 393–402. doi: 10.1111/j.1469-8137.2010.03410.x

Parachnowitsch, A. L., Manson, J. S., and Sletvold, N. (2019). Evolutionary ecology of nectar. *Ann. Bot.* 123, 247–261. doi: 10.1093/aob/mcy132

Parachnowitsch, A. L., Raguso, R., and Kessler, A. (2012). Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated *Penstemon digitalis*. *New Phytol.* 195, 667–675. doi: 10.1111/j.1469-8137.2012.04188.x

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* 170, 23–40. doi: 10.1007/BF00190398

Phillips, P. C., and Arnold, S. J. (1989). Visualizing multivariate selection. *Evolution* 43, 1209–1222. doi: 10.1111/j.1558-5646.1989.tb02569.x

Pohl, M., Watolla, T., and Lunau, K. (2008). Anther-mimicking floral guides exploit a conflict between innate preferences and learning in bumblebees (*Bombus terrestris*). *Behav. Ecol. Sociobiol.* 63, 295–302. doi: 10.1007/s00265-008-0661-x

Price, G. R. (1970). Selection and covariance. *Nature* 227, 520–521. doi: 10.1038/227520a0

Pyke, G. H. (2016). Floral nectar: pollinator attraction or manipulation? *Trends Ecol. Evol.* 31, 339–341. doi: 10.1016/j.tree.2016.02.013

Rausher, M. D. (2008). Evolutionary transitions of flower color. *Int. J. Plant Sci.* 169, 7–21. doi: 10.1086/523358

Renoult, J. P., Thomann, M., Schaefer, H. M., and Cheptou, P. O. (2013). Selection on quantitative colour variation in *Centaurea cyanus*: the role of the pollinator's visual system. *J. Evol. Biol.* 26, 2415–2427. doi: 10.1111/jeb.12234

Rodriguez-Castañeda, N. L., Ortiz, P. L., Arista, M., Narbona, E., and Buide, M. L. (2020). Indirect selection on flower color in *Silene littorea*. *Front. Plant Sci.* 11:588383. doi: 10.3389/fpls.2020.588383

Rohde, K., Papiorek, S., and Lunau, K. (2013). Bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) prefer similar colours of higher spectral purity over trained colours. *J. Comp. Physiol. A* 199, 197–210. doi: 10.1007/s00359-012-0783-5

Salzmann, C. C., Cozzolino, S., and Schiestl, F. P. (2007). Floral scent in food-deceptive orchids: species specificity and sources of variability. *Plant Biol.* 9, 720–729. doi: 10.1055/s-2007-965614

Sapir, Y., Gallagher, M. K., and Senden, E. (2021). What maintains flower colour variation within populations? *Trends Ecol. Evol.* 36, 507–519. doi: 10.1016/j.tree.2021.01.011

Schiestl, F. P., and Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* 28, 307–315. doi: 10.1016/j.tree.2013.01.019

Shrestha, M., Dyer, A. G., and Burd, M. (2013). Evaluating the spectral discrimination capabilities of different pollinators and their effect on the evolution of flower colours. *Commun. Integr. Biol.* 6:e24000. doi: 10.4161/cib.24000

Shrestha, M., Garcia, J. E., Chua, J. H. J., Howard, S. R., Tscheulin, T., Dorin, A., et al. (2019). Fluorescent pan traps affect the capture rate of insect orders in different ways. *Insects* 10:40. doi: 10.3390/insects10020040

Skorupski, P., Spaethe, J., and Chittka, L. (2006). Visual search and decision making in bees: time, speed, and accuracy. *Int. J. Comp. Psychol. A* 19, 342–357.

Sletvold, N. (2019). The context dependence of pollinator-mediated selection in natural populations. *Int. J. Plant Sci.* 180, 934–943. doi: 10.1086/705584

Sletvold, N., and Ågren, J. (2014). There is more to pollinator-mediated selection than pollen limitation. *Evolution* 68, 1907–1918. doi: 10.1111/evo.12405

Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. *Evolution* 70, 716–724. doi: 10.1111/evo.12881

Sobral, M., Veiga, T., Domínguez, P., Guitián, J. A., Guitián, P., and Guitián, J. M. (2015). Selective pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS One* 10:e0132522. doi: 10.1371/journal.pone.0132522

Souto-Vilarósa, D., Vuletaa, A., Manitašević Jovanovič, S., Budečević, S., Wang, H., Sapir, Y., et al. (2018). Are pollinators the agents of selection on flower colour and size in irises? *Oikos* 127, 834–846. doi: 10.1111/oik.04501

Souza, C. S., Maruyama, P. K., Aoki, C., Sigrist, M. R., Raizer, J., Gross, C. L., et al. (2018). Temporal variation in plant-pollinator networks from seasonal tropical environments: higher specialization when resources are scarce. *J. Ecol.* 106, 2409–2420. doi: 10.1111/1365-2745.12978

Stavenga, D. G., Staal, M., and van der Kooi, C. J. (2020). Conical epidermal cells cause velvety colouration and enhanced patterning in *Mandevilla* flowers. *Faraday Discuss.* 223, 98–106. doi: 10.1039/D0FD00055H

Stevens, M., Párraga, C. A., Cuthill, I. C., Partridge, J. C., and Troscianko, T. S. (2007). Using digital photography to study animal coloration. *Biol. J. Linn. Soc.* 90, 211–237. doi: 10.1111/j.1095-8312.2007.00725.x

Stinchcombe, J. R., Agrawal, A. F., Hohenlohe, P. A., Arnold, S. J., and Blows, M. W. (2008). Estimating nonlinear selection gradients using quadratic regression coefficients: double or nothing? *Evolution* 62, 2435–2440. doi: 10.1111/j.1558-5646.2008.00449.x

Strauss, S. Y., and Whittall, J. B. (2006). "Non-pollinator agents of selection on floral traits," in *Ecology and Evolution of Flowers*, eds L. D. Harder and S. C. H. Barrett (Oxford: Oxford University Press).

Suzuki, M. F., and Ohashi, K. (2014). How does a floral colour-changing species differ from its non-colour-changing congener? - a comparison of trait combinations and their effects on pollination. *Funct. Ecol.* 28, 549–560. doi: 10.1111/1365-2435.12209

Telles, F. J., and Rodríguez-Gironés, M. A. (2015). Insect vision models under scrutiny: what bumblebees (*Bombus terrestris L.*) can still us. *Sci. Nat.* 102:4. doi: 10.1007/s00114-014-1256-1

Tong, H., Li, J., Wo, Y., Shao, G., Zhao, W., Aguilar-Gómez, D., et al. (2019). Effects of substrate color on intraspecific body color variation in the toad-headed lizard, *Phrynocephalus versicolor*. *Ecol. Evol.* 9, 10253–10262. doi: 10.1002/ece3.5545

Troje, N. (1993). Spectral categories in the learning behaviour of blowflies. *Z. Naturforsch.* 48, 96–104. doi: 10.1515/znc-1993-1-218

Trunschke, J., Sletvold, N., and Ågren, J. (2017). Interaction intensity and pollinator-mediated selection. *New Phytol.* 214, 1381–1389. doi: 10.1111/nph.14479

Trunschke, J., Sletvold, N., and Ågren, J. (2020). Manipulation of trait expression and pollination regime reveals the adaptive significance of spur length. *Evolution* 74, 597–609. doi: 10.1111/evo.13927

Vaknin, Y., Tov, Y. Y., and Eisikowitch, D. (1996). Flowering seasonality and flower characteristics of *Loranthus acacia* Zucc. (Loranthaceae): implications for advertisement and bird-pollination. *Sex. Plant Reprod.* 9, 279–285. doi: 10.1007/s004970050044

van der Kooi, C. J., Dyer, A. G., Kevan, P. G., and Lunau, K. (2019). Functional significance of the optical properties of flowers for visual signaling. *Ann. Bot.* 123, 263–276. doi: 10.1093/aob/mcy119

van der Kooi, C. J., Elzenga, J. T. M., Staal, M., and Stavenga, D. G. (2016). How to colour a flower: on the optical principles of flower coloration. *Proc. R. Soc. B* 283:9. doi: 10.1098/rspb.2016.0429

Van der Niet, T., Peakall, R., and Johnson, S. D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Ann. Bot.* 113, 199–211. doi: 10.1093/aob/mct290

van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belušič, G., and Kelber, A. (2021). Evolution of insect color vision: from spectral sensitivity to visual ecology. *Annu. Rev. Entomol.* 66, 435–461. doi: 10.1146/annurev-ento-061720-071644

Veiga, T., Guitián, J., Guitián, P., Guitián, J., Munilla, I., and Sobral, M. (2015). Flower color variation in the montane plant *Gentiana lutea* L. (Gentianaceae) is unrelated to abiotic factors. *Plant Ecol. Div.* 9, 105–112. doi: 10.1080/17550874.2015.1074626

Verhoeven, C., Ren, Z.-X., and Lunau, K. (2018). False-colour photography: a novel digital approach to visualize the bee view of flowers. *J. Pollinat. Ecol.* 23, 102–118. doi: 10.26786/1920-7603(2018)11

Vignolini, S., Moyroud, E., Glover, B. J., and Steiner, U. (2013). Analysing photonic structures in plants. *J. R. Soc. Interf.* 10:20130394. doi: 10.1098/rsif.2013.0394

von Helversen, O. (1972). Zur spektralen unterschiedsempfindlichkeit der honigbiene. *J. Comp. Physiol. A* 80, 439–472. doi: 10.1007/BF00696438

Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B., and Menzel, R. (2001). Colour thresholds and receptor noise: behaviour and physiology compared. *Vision Res.* 41, 639–653. doi: 10.1016/S0042-6989(00)00288-1

Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. B* 265, 351–358. doi: 10.1098/rspb.1998.0302

Waser, N. M., and Price, M. V. (1981). Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* 35, 376–390. doi: 10.1111/j.1558-5646.1981.tb04896.x

Wassink, E., and Caruso, C. M. (2013). Effect of coflowering *Mimulus ringens* on phenotypic selection on floral traits of gynodioecious *Lobelia siphilitica*. *Botany* 91, 745–751. doi: 10.1139/cjb-2013-0112

Weiss, M. R. (1995). Floral color change: a widespread functional convergence. *Am. J. Bot.* 82, 167–185. doi: 10.1002/j.1537-2197.1995.tb11486.x

Yabuya, T., Saito, M., Iwashina, T., and Yamaguchi, M. (2000). Stability of flower colors due to anthocyanin-flavone copigmentation in Japanese garden iris, *Iris ensata* Thunb. *Euphytica* 115, 1–5. doi: 10.1023/A:1003916417168

Zhang, G., Meng, L., Wu, Z., Zhang, Z., Yin, L., Yang, Y., et al. (2017). Natural selection on floral traits of *Caltha scaposa* (Ranunculaceae), an alpine perennial with generalized pollination system from Northwest Yunnan. *Plant Div.* 39, 202–207. doi: 10.1016/j.pld.2017.03.001

Zvi, M. M. B., Negre-Zakharov, F., Masci, T., Ovadis, M., Shklarman, E., Ben-Meir, H., et al. (2008). Interlinking showy traits: co-engineering of scent and colour biosynthesis in flowers. *Plant Biotechnol. J.* 6, 403–415. doi: 10.1111/j.1467-7652.2008.00329.x

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